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**AN ASSESSMENT OF FACTORS AFFECTING FREE RADICAL
FORMATION IN INFANTS RECEIVING PARENTERAL NUTRITION**

Submitted for the degree of Master of Surgery in the
Faculty of Medicine of the University of London

2002

RAJAT KUMAR BASU

DEPARTMENT OF SURGERY
INSTITUTE OF CHILD HEALTH, LONDON

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ABSTRACT

The development of parenteral nutrition has been a major advance in the care of neonates undergoing surgery. However, long-term parenteral nutrition can result in complications, some of which may result from an increased free radical activity associated with the lipid component of the regimen.

This thesis describes studies undertaken to determine the effects of total parenteral nutrition (TPN) on free radical formation in neonatal surgical patients. The parenteral nutrition regimen was then modified in an attempt to reduce free radical formation.

Lipid peroxides and malondialdehyde (MDA), some of the products of free radical formation, were assayed in parenteral nutrition solutions. The mean lipid peroxide concentration was $19.25\mu\text{mol/l}$ and the mean MDA concentration was $8.69\mu\text{mol/l}$. Neonates receiving TPN had significantly greater MDA concentrations than those not receiving TPN ($p<0.0001$). Stopping the lipid infusion resulted in a significant decrease in the mean plasma MDA concentration ($p<0.01$). Promoting oxidation of the administered lipid by reducing the carbohydrate to lipid ratio of the TPN also resulted in significantly decreased plasma MDA concentrations ($p<0.01$). The administration of a medium chain triglyceride emulsion was, however, associated with significantly greater plasma MDA concentrations than when a long chain triglyceride

emulsion was used ($p < 0.05$). Partial enteral feeding during parenteral nutrition did not have any significant effect on free radical activity.

A relationship between free radical activity and illness severity in sick neonates was sought. There was no significant correlation between plasma MDA concentrations and the Paediatric Risk of Mortality (PRISM) score. However MDA concentrations showed a positive correlation with plasma TNF- α ($p < 0.05$) and IL-6 ($p < 0.001$) concentrations.

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ABBREVIATIONS

ATP	Adenosine triphosphate
cal	Calories
C.V.	Coefficient of variation
EE	Energy expenditure
ELISA	Enzyme linked immunosorbent assay
FOX	Ferrous oxidation xylenol orange
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidase
IL	Interleukin
i.v.	Intravenous
LCT	Long chain triglyceride
LOOH	Lipid hydroperoxide
MCT	Medium chain triglyceride
MDA	Malondialdehyde
NAD	Nicotinamide adenine dinucleotide
NICU	Neonatal intensive care unit
NEC	Necrotizing enterocolitis
PG	Prostaglandin
PRISM	Paediatric risk of mortality
PT	Prothrombin time
PTT	Partial thromboplastin time
s.d.	Standard deviation
TBARS	Thiobarbituric acid reactive substances

TNF	Tumour necrosis factor
TPN	Total parenteral nutrition
TPP	Triphenylphosphine
•	Denotes a free radical

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STATEMENT OF ORIGINALITY

All aspects of the clinical studies were performed by the author, including patient selection and recruitment, prescribing the TPN and performing indirect calorimetry, blood and breath sampling.

The laboratory analysis of the blood samples for MDA and lipid peroxides was performed by the author.

The laboratory analysis of the blood samples for TNF- α and IL-6 was performed by Miss E. Papp.

The breath sampling equipment was set up and the breath samples collected by the author. The samples were analysed by Mrs J. Drury at the Regional Neonatal Unit, Liverpool Women's Hospital.

PUBLICATIONS ARISING FROM THIS THESIS

Lipid peroxidation can be reduced in infants on TPN by promoting fat utilization.

R Basu, I Merryweather, S Eaton, DPR Muller, A Pierro

Journal of Pediatric Surgery 1999 Feb; 34(2): 255-9.

Free radical formation in infants: the effect of critical illness, parenteral nutrition and enteral feeding.

R Basu, DPR Muller, E Papp, S Eaton, N Klein, A Pierro

Journal of Pediatric Surgery 1999 Jul; 34(7): 1091-1095.

Energy metabolism of infants and children with systemic inflammatory response syndrome and sepsis.

Turi RA, Petros AJ, Eaton S, Fasoli L, Basu R, Spitz L, Pierro A

Annals of Surgery 2001 Apr; 233(4): 581-7.

BACKGROUND

BACKGROUND

1.1 INTRODUCTION

The development of total parenteral nutrition (TPN) over the last 30 years has transformed the care of neonates and infants undergoing surgery. The energy stores of this group of patients are sufficient for only a few days, but normal food intake and absorption may be disordered for many days or weeks. The development of lipid infusions represents one of the landmarks in the development of TPN. A solution of low osmolality, intravenous lipid delivers a large quantity of calories in a very small volume and is the only source of essential fatty acids. However intravenous lipid is thought to be the source of some of the complications of TPN.

Some of the complications of lipid infusions may arise due to increased free radical formation. Evidence for raised free radical activity during TPN administration was first noted by Wispe in 1985 and confirmed by other groups (Van Gossum et al 1988; Pitkanen et al 1991).

Slater described a free radical mechanism for CCl₄ hepatotoxicity in 1966. Since this first description, free radicals have been implicated in the pathogenesis of a range of diseases, including some neonatal disorders. Premature neonates are a vulnerable group of patients in many respects, including an increased susceptibility to oxidative stress. Oxygen therapy is frequently required and TPN is often used for nutrition until feeding is established.

I wished to investigate the extent to which infants are exposed to oxidative stress due to TPN administration. I also wished to find ways of

reducing free radical formation during TPN so that this essential therapy may be used more safely.

This thesis describes clinical studies which were designed to assess the extent of free radical formation during TPN and some measures which may be used in clinical practice to ameliorate this problem.

Although free radical activity has been implicated in the pathogenesis of many diseases, there are no studies testing whether the extent of free radical activity bears a relation to measures of severity of illness. For the first time the relationship between free radical formation and illness severity was explored and correlated with indices of illness severity. The possible implications of my results for clinical practice are discussed.

1.2 FREE RADICALS

1. Definition

Atoms, whether they exist alone or in combination with other atoms in a molecule, consist of a positively charged nucleus surrounded by negatively charged electrons occupying orbitals around the nucleus. An orbital can hold a maximum of two electrons and the atom is most stable when its electrons are in pairs. When a single electron occupies an orbital, the electron is unpaired. A free radical is an atom or molecule in possession of an unpaired electron. Electron pairing does not necessarily relate to the total number of electrons present, therefore a radical may be electrically neutral, or carry a positive or negative charge (Punchard & Kelly 1996).

Free radicals can be formed if a non radical gains or loses a single electron. Radicals are also created if a covalent bond is broken and one electron from the shared pair accompanies each atom (homolytic fission). Many free radicals are highly reactive, others are relatively stable. When a free radical reacts with a non-radical it does so either by donating or by removing an electron. This creates another free radical, which may be more or less reactive than the first.

2 *Oxygen derived free radicals*

Molecular oxygen (O_2) is a free radical, having two unpaired electrons. Each group of orbitals or “shells” has a quantum number 1, 2, 3...etc. Sub-shells are denoted by letters s, p, d, f. The s sub-shell can hold two electrons. The p sub-shell has three orbitals. Each sub-shell may hold two electrons, but at a

given energy level each orbital receives one electron before any receives two (Hund's rule). The electrons in a pair have opposite spins. Electrons in an orbital with opposite spins may be denoted with arrows pointing in opposite directions. The arrangement of the sixteen electrons of molecular oxygen in their sub-shells can be illustrated as follows:

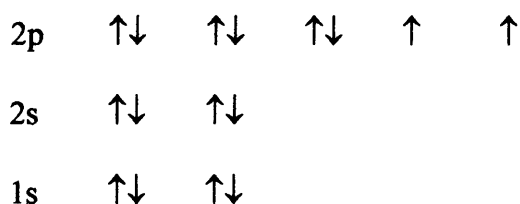


Figure 1.2.1 A simplified representation of the arrangement of electrons of molecular oxygen. Each orbital in each sub-shell receives one electron before any receives two, therefore there are unpaired electrons with parallel spins in the 2p sub-shell.

Molecular oxygen may be considered a free radical because there are two unpaired electrons in the 2p sub-shell. O_2 is relatively stable in comparison with most free radicals because of the parallel spins of the unpaired electrons. This means that if O_2 is to accept a pair of electrons then they must be of antiparallel spins to fill the spaces in the 2p sub-shell. This restriction reduces the reactivity of oxygen (Halliwell & Gutteridge 1999, p.24).

Other oxygen derived radicals resulting from the reduction of oxygen, or from homolytic fission of oxygen containing compounds, include the superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical (OH^{\cdot}), and the alkoxyl radical (RO^{\cdot}). The

term “reactive oxygen species” is used to describe these radicals and other oxygen derived, non-radical compounds including hydrogen peroxide (H₂O₂).

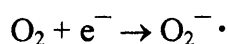
3. *Physiological generation of free radicals*

The role of free radicals in the pathogenesis of many disorders, and the potential preventive or therapeutic value of antioxidants, has received much scientific and popular attention. However free radicals play an important role in normal physiology, both as intermediates in chemical reactions and as active molecules in their own right. Some of these roles are outlined below.

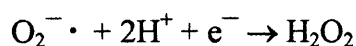
4. *Physiological generation of free radicals: the electron transport chain*

The most important mechanism for the production of adenosine triphosphate (ATP) in aerobic organisms is oxidative phosphorylation - a process whereby the energy derived from the oxidation of food is utilized in the phosphorylation of adenosine diphosphate (ADP). During this process oxygen accepts electrons as it is reduced to water and ADP is converted to ATP. The reactions are regulated by the respiratory chain enzymes of the inner mitochondrial membrane

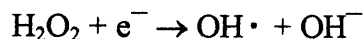
Reduction of O₂ by the addition of one electron produces the superoxide anion (O₂^{-•}):



Hydrogen peroxide results from the addition of two protons and a further electron:



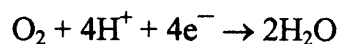
With the addition of a further electron the hydroxyl radical and hydroxyl ion are produced:



These combine with a proton and an electron and a proton respectively to produce water:

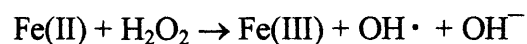


The overall reaction is therefore:



The protons and electrons in these reactions are derived from nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD) hydrogen carriers, which are themselves reduced as part of the oxidation of glucose and fatty acids. Thus the superoxide anion ($\text{O}_2^{\bullet -}$) and the hydroxyl radical (OH^\bullet) are produced during the reduction of oxygen. Cytochrome oxidase normally keeps these free radical intermediates bound to the reaction site (Halliwell 1984), but leakage of electrons may occur (Punchard & Kelly 1996), resulting in superoxide formation, which may interact with cellular molecules.

Hydrogen peroxide is also generated. On its own H_2O_2 is relatively un-reactive. However H_2O_2 readily crosses cell membranes and can interact with iron and copper ions to produce species such as the hydroxyl radical



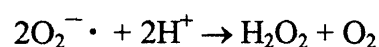
5. *Physiological generation of free radicals: phagocytic cells*

Oxygen derived free radicals are produced during bacterial killing by phagocytic white blood cells. Circulating white blood cells, in particular, neutrophils and monocytes, leave the circulation and migrate to areas of inflammation under the influence of chemotactic factors. Once they are in an inflamed area monocytes differentiate into macrophages, which have increased motility and phagocytic capacity. Infecting bacteria become coated with immunoglobulins and complement components. Neutrophils and macrophages have receptors for these proteins, which enable them to recognise the bacteria and which trigger phagocytosis. Activated neutrophils and macrophages show a large increase in oxygen metabolism which has been termed the “respiratory burst”. The increased oxygen uptake (ten to twenty times the resting oxygen utilization) is not due to an increase in respiration, but to the production of superoxide by activated enzymes on the cell membrane.

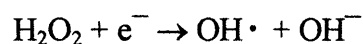
The membrane enzymes reduce oxygen by electron transfer from nicotinamide adenine dinucleotide phosphate (NADPH, generated by the pentose phosphate pathway, Lunec 1990):



Phagocytosed bacteria are therefore engulfed in a membrane producing the superoxide anion. Superoxide reacts with protons to form hydrogen peroxide:



The hydrogen peroxide may give rise to the hydroxyl radical:



The reactive oxygen species are toxic to ingested bacteria (Punchard & Kelly 1996).

In chronic granulomatous disease there is a failure of the neutrophil respiratory burst. This rare inherited immune disorder results in increased susceptibility to certain bacterial and fungal infections due to the absence of neutrophil free radical production.

6. Physiological generation of free radicals: prostaglandin and leukotriene synthesis

Prostaglandins (PG) and leukotrienes are important mediators in the inflammatory response. They are produced from the 20-carbon fatty acid arachidonic acid, via oxidation reactions (catalysed by cyclooxygenase and lipoxygenase) which proceed via free radical intermediates. The conversion of PGG₂ to PGH₂ results in radical formation (Lands 1979). Furthermore, leukotrienes stimulate the respiratory burst of neutrophils.

7. Physiological generation of free radicals: nitric oxide

Nitric oxide (NO) is generated from the amino acid L-arginine by the enzyme NO synthase. NO has an unpaired electron and is therefore a free radical. It is soluble in water and in organic solvents, therefore can move readily within and between cells. NO has been found to be active in three main areas of physiology - the vascular system, the immune system and the nervous system (Vallance & Collier 1994).

NO synthesised by vascular endothelial cells produces a constant background vasodilator tone and may therefore play a central role in the

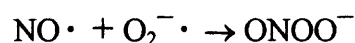
regulation of blood flow and blood pressure. Nitrates used therapeutically for angina pectoris (e.g. glyceryl trinitrate) exert their effects because they are NO donors. In neonatology the vasodilator effect of inhaled NO is used to treat pulmonary hypertension (Mupanemunda & Edwards 1995).

In the immune system NO is produced by activated macrophages and has been found to be increased in many inflammatory disorders. NO alone or in combination with oxygen derived free radicals is toxic to bacteria, fungi and parasites, as previously described.

NO acts as a neurotransmitter for peripheral nerves supplying the smooth muscle of blood vessels, the bronchi, the gut and the urinary tract. NO has also been identified as a neurotransmitter in the central nervous system.

A deficiency of NO has been implicated in the pathogenesis of many diseases. In paediatric surgery, a reduction in the proportion of nerves expressing nitric oxide synthase has been found on samples of pyloric muscle in infants with hypertrophic pyloric stenosis (Abel et al 1998).

As a free radical NO may be toxic to cells by damaging metabolic enzymes (e.g. ribonucleotide reductase) or by reacting with other free radicals to produce more toxic species, e.g. with superoxide to form peroxynitrite (ONOO^-):



Peroxynitrite is a potent oxidant which causes tissue damage via reactions with protein and non-protein –SH groups (Halliwell & Gutteridge 1999, p. 78).

8. *Antioxidants: defences against free radicals*

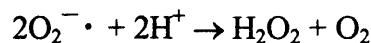
For aerobic organisms, oxygen is essential for life, but because of free radical production, it is potentially toxic to the organism. In order to limit the toxicity, antioxidants are present which remove free radicals or prevent their overproduction.

Antioxidants may be classified as intracellular or extracellular, water soluble or lipid soluble and enzymic or non-enzymic. Their antioxidant properties are manifest in a variety of ways (Punchard & Kelly 1996).

i) Direct reaction with free radicals:

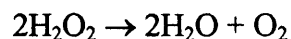
e.g. α -tocopherol (the most active form of vitamin E) reacts with lipid peroxy radicals ($\text{LOO} \cdot$) preventing the propagation of lipid peroxidation.

ii) The conversion of superoxide ($\text{O}_2^{\cdot -}$) to hydrogen peroxide is greatly accelerated by the enzyme superoxide dismutase (SOD):

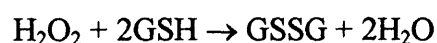


iii) The detoxification of hydrogen peroxide (a precursor of free radicals):

e.g. a) catalases catalyse the decomposition of hydrogen peroxide to water and oxygen:



b) the glutathione peroxidase family of enzymes couple the reduction of hydrogen peroxide with the conversion of reduced glutathione (GSH) to its oxidised form, a compound composed of two glutathione molecules linked by a disulphide bridge (GSSG), by oxidation of the SH group of cysteine:



iii) Binding of metal ions which act as catalysts for free radical production: e.g. the iron binding protein transferrin.

9. Oxidative stress

Oxidative stress is defined as a disturbance of the pro-oxidant and antioxidant balance in favour of the former. The imbalance can occur as a result of a surplus of free radicals or a deficiency of the antioxidant defences.

Increased generation of free radicals occurs:

- i) via endogenous, physiological, mechanisms (e.g. the electron transport chain)
- ii) due to physical stimuli (e.g. ionising radiation)
- iii) due to toxins (e.g. CCl₄, Slater 1966)
- iv) caused by mechanical production (e.g. bone fracture, Symons 1996)
- v) during infective and inflammatory disorders (the phagocytic respiratory burst).

10. Free radicals: the pathogenesis of disease

Free radical mechanisms have been proposed as part of the pathogenesis of a wide range of diseases. It is thought that free radicals exert their pathogenic effects by reacting with cellular DNA, proteins and lipids, destroying the molecules or disrupting their structure and therefore their function.

The reaction with lipids has received particular attention in this respect. Lipid molecules are abundant in living systems as the major component of cell membranes. The products of free radical attack upon lipids have been identified and measured in many pathological states (Halliwell & Chirico 1993). Free radical attack upon an unsaturated fatty acids begins with hydrogen abstraction

from the $-\text{CH}_2-$ group of the side chain. This creates a carbon centred radical, $-\text{C}\cdot\text{H}-$, (Figure 1.2.2). In aerobic conditions this radical reacts with O_2 to form a peroxy radical, $-\text{COO}\cdot$. This will stabilise itself at the expense of another nearby molecule, abstracting hydrogen to create another radical, the first molecule forming a lipid peroxide, LOOH . Thus once the first fatty acid radical has been formed, the process becomes a self propagating chain reaction. Every cell in the body is potentially vulnerable, having a membrane composed of unsaturated fatty acids, oxygen for respiration, and catalysts such as iron in cytochromes or haemoglobin. The altered structure of the fatty acid may have a deleterious effect upon the function of the membrane, reducing its fluidity, for example. In addition, the lipid peroxides or their breakdown products may be toxic to cellular proteins (Dormandy 1969; Richter 1987).

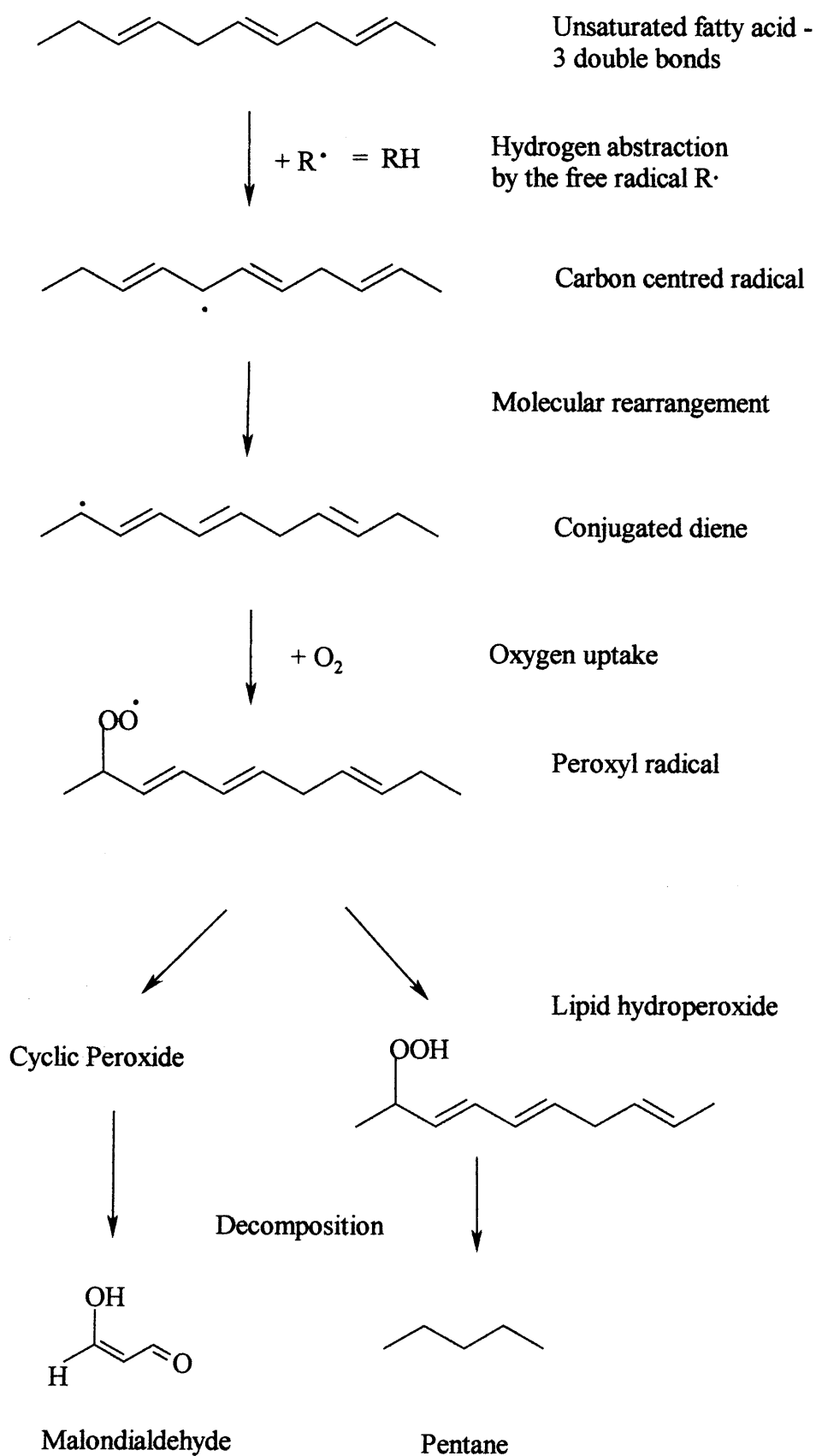


Figure 1.2.2 Lipid peroxidation and decomposition consequent upon free radical attack

11. Free radical disorders of neonatology

Premature neonates are prone to disorders associated with oxidative stress because of:

- i) increased formation of free radicals (e.g. from O₂ therapy) and
- ii) a relative deficiency of antioxidant defences (e.g. the newborn has relatively low levels of vitamin E due to a reduced concentration of its transport molecule, β -lipoprotein) (Haga et al 1982).

Four conditions which primarily affect premature infants have been linked to free radical mediated reactions, namely bronchopulmonary dysplasia, retinopathy of prematurity, intraventricular haemorrhage, and necrotizing enterocolitis (NEC) (Rice-Evans & Gopinathan 1995; Saugstad 1996).

Bronchopulmonary dysplasia is a chronic lung disease of premature infants who have received high concentrations of inspired O₂ and prolonged mechanical ventilation for respiratory distress syndrome, meconium aspiration, persistent fetal circulation, or congenital heart disease. The pathogenesis of the disorder is multifactorial, and includes lung immaturity (surfactant deficiency and impaired mucociliary function), barotrauma and infection. High concentrations of inspired oxygen and reduced lung antioxidant defences predispose the neonatal lung to tissue damage caused by the superoxide anion (O₂⁻) and other oxygen derived free radicals (Evans et al 1986, Saugstad 1997).

Retinopathy of prematurity is a disorder in which ischaemia of the eye is followed by abnormal blood vessel proliferation in the retina. This may cause haemorrhage, scarring & retinal detachment. The pathogenesis of the disorder is multifactorial, but oxygen therapy is a major risk factor. Other risk factors include low birth weight, hypoxia and hypercarbia and hypocarbia. In addition

antioxidant levels in the premature retina may be low which would further contribute to the potential for oxygen induced injury (Ben-Sira 1988).

Intraventricular haemorrhage is bleeding into the subependymal germinal matrix which occurs almost exclusively in premature babies within the first few weeks of life. The germinal matrix is a primitive cellular region located ventro-lateral to the lateral ventricle at the level of the foramen of Monro. It is quite prominent at 26 to 32 weeks of gestation and involutes by term. Intraventricular haemorrhage occurs during involution of thin walled vessels of the matrix overlying the head of the caudate nucleus. The haemorrhage may remain confined to the germinal matrix or rupture into the ventricles. Bleeding from the choroid plexus may also contribute to the ventricular blood. It is a major cause of death in low birthweight infants and survivors may have varying degrees of neurological abnormality. The pathogenesis of intraventricular haemorrhage is multifactorial and includes platelet and coagulation disturbances and immaturity of the vessels of the germinal matrix (making them more likely to rupture). Fluctuating blood flow to the brain may result in ischaemia and reperfusion episodes which are associated with free radical mediated cellular damage (Grace 1994).

NEC is the most common acquired neonatal gastrointestinal surgical emergency. It is a multifactorial disease characterised by oedema, haemorrhage and necrosis of the gut most frequently affecting the terminal ileum, caecum and ascending colon. In severe cases extensive regions of the small and large intestines may be involved, with transmural necrosis and perforation. The initiating events in the pathogenesis of NEC have not been conclusively established, but epidemiological studies have implicated the potential

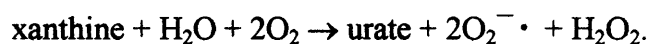
contributory roles of infection, enteric feeding, and local gastrointestinal vascular compromise. Ischaemia and reperfusion injury and the local inflammatory response are both routes by which free radical mechanisms may play a role in the pathogenesis of the disorder (Clark et al 1988). The possible therapeutic role of vitamin E has been investigated in an experimental rat model of NEC, induced by ischaemia and reperfusion injury (Okur et al 1995). The group treated with vitamin E showed milder histological changes and had lower evidence of lipid peroxidation than the untreated group.

12. Ischaemia and reperfusion injury

Ischaemia occurs when interference with the blood supply to an organ or part of the body results in tissue hypoxia. It may result from blockage of the arterial inflow (as a result of atherosclerosis or thrombo-embolic disease) or because of a deficiency of perfusion (cardiac failure, hypovolaemia). Different tissues are able to withstand differing periods of hypoxia before irreversible cell damage results. If the circulation to the tissue is restored within the time necessary for that tissue, recovery from the hypoxia can occur. However the restoration of blood flow to an ischaemic tissue can initially be accompanied by further local injury. There is evidence that this injury is in part due to free radical species generated by the enzyme xanthine oxidase during reperfusion (McCord 1985).

The enzyme xanthine oxidase exists as xanthine dehydrogenase in healthy tissue, where it catalyses the conversion of xanthine to uric acid and transfers electrons to NAD^+ to form NADH. Ischaemia causes the conversion of xanthine dehydrogenase to xanthine oxidase. This is thought to occur as a result

of a calcium activated protease (McCord 1985). During hypoxic episodes ion pumps which maintain normal calcium levels no longer function, therefore intracellular calcium levels increase. Hypoxia also results in the accumulation of one of the substrates for the enzyme - hypoxanthine. The production of adenosine triphosphate (ATP) is dependent upon an adequate oxygen supply. During ischaemia therefore, ATP production decreases and the ATP present is used up. The resulting adenosine monophosphate (AMP) is catabolized to hypoxanthine. Xanthine and hypoxanthine are both substrates for xanthine oxidase in the following reaction:



Thus on reperfusion, this reaction proceeds and oxygen is converted to superoxide radicals. This mechanism may be responsible for reperfusion injury following gut and brain ischaemia and so be implicated in the pathogenesis of NEC and intraventricular haemorrhage (Saugstad 1996).

13. Measuring free radical activity

Free radicals are extremely reactive and therefore short lived. The only technique that directly measures free radical activity is electron spin resonance spectrometry. This technique utilizes the phenomenon of resonance displayed by an unpaired electron when placed in a magnetic field. However only relatively high, steady state concentrations of free radicals can be measured. Compounds which react with free radicals to form stable adducts (called “spin traps”) can be used to increase the applicability of the technique. However electron spin

resonance spectrometry remains impractical for use in vivo and has a limited role for blood or tissue samples (Holley & Cheeseman 1993).

A more useful approach in biomedical practice is the measurement of the products of free radical reactions, also referred to as the “footprints” of free radical activity. The “footprints” left by free radicals are the products formed from their reactions with DNA, proteins and, most commonly, lipids.

The polyunsaturated lipid component of cell membranes is particularly vulnerable to attack by free radicals at the site of the double bond in the fatty acid chain. The mechanism of attack initiates a chain reaction, as discussed earlier, leading to the destruction of membrane lipids and the formation of lipid peroxides (Halliwell & Chirico 1993).

Decomposition of lipid peroxides results in the formation of a variety of products including malondialdehyde (MDA) and pentane (Figure 1.2.2). Lipid peroxides and their degradation products are among the most commonly measured indices of free radical activity.

Ischaemia reperfusion injury – additional comments

Ischaemia reperfusion injury (IRI) may be defined as cell dysfunction, necrosis or apoptosis following hypoxia and re-oxygenation. The injury results, in part, from cellular adaptive changes to hypoxia that then are harmful to the cell when oxygen is again available. Some of these changes or the cell damage that results from them have mechanisms relating to free radical formation.

Granger (1981) and McCord (1985) were first to propose the role of superoxide radicals, generated by xanthine oxidase (XO), in causing cell damage in IRI. Xanthine oxidase exists in two forms, xanthine oxidase and xanthine dehydrogenase (XD). Both forms catalyze the conversion of hypoxanthine to xanthine and xanthine to uric acid in purine degradation. The gene for the enzyme is on chromosome 2. Transcription has been found to be up-regulated by hypoxia, lipopolysaccharide, interferon gamma, interleukins, TNF & cortisol - i.e. transcription is increased in response to stress or infection (Berry 2004).

The enzyme is mostly in the XD form, but is reversibly converted to the XO form under anaerobic conditions and irreversibly converted to XO by proteases. When hypoxanthine and xanthine are oxidised, the XO form preferentially transfers electrons to oxygen, generating superoxide and hydrogen peroxide. As described, superoxide reduces Fe(III) to Fe(II), which reacts with hydrogen peroxide to form the highly reactive hydroxyl radical, which may then go on to cause lipid peroxidation or other molecular damage. Support for this sequence of events comes from studies showing that the xanthine oxidase inhibitor allopurinol and iron chelator deferoxamine decrease some of the deleterious consequences of IRI (Peeters-Scholte 2003).

However xanthine oxidase, nucleotides and free radicals may interact in a

more complex and extensive way during IRI. A range of reactive oxygen and nitrogen species have been implicated in causing post-hypoxic cellular injury and these free radicals may act on intracellular signaling systems to cause apoptosis rather than behaving simply as reactants that damage membrane lipids, DNA, or proteins (Li 2002). Apoptosis is a process of cellular self-destruction that results in the formation of an apoptotic body. This differs from necrosis when rupture of the cell results in the release of cytoplasmic contents and the consequently an inflammatory response. The apoptotic regulatory pathways converge on a common effector mechanism of cell death orchestrated by a family of proteases called caspases. During ischaemia it has been shown that xanthine increases apoptosis and caspase activity, probably via the action of reactive oxygen species (Genesca 2002).

Free radical production during ischaemia may also occur from mechanisms other than the xanthine oxidase mediated reaction. The activity of the enzymes in mitochondrial electron transfer chain (e.g. cytochrome oxidase and manganese superoxide dismutase) decrease during hypoxia. Loss of cytochrome oxidase activity leads to cellular injury during reoxygenation, because absence of the final electron acceptor increases ROS production by more proximal complexes.

In summary, a number of interrelated mechanisms involving xanthine oxidase, free radical production, down-regulation of mitochondrial electron transfer chain enzymes, changes in cell signalling pathways and down-regulation of antioxidant defences have been implicated in causing ischaemia reoxygenation injury. Research continues to aim to exploit this knowledge to reduce IRI and its consequences.

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1.3 TOTAL PARENTERAL NUTRITION

1. Introduction

Parenteral nutrition refers to the delivery of nutrients by the intravenous route. It has grown from its infancy to a successful form of therapy during the last 30 years. During this time stable, sterile and nutritionally appropriate feeds have been developed and materials have become available which permit long term venous access.

2. Early experiments

A description of the intravenous infusion of wine is first credited to Sir Christopher Wren. His experiments are described in volume I of the Philosophical Transactions of the Royal Society of London, 1665. From William Harvey's description of the circulation of the blood, it was known that the intravenous infusion would flow towards the heart and then to the brain. Therefore a ligature was tied around a large vein in the hind leg of a dog and a cannula (in the form of a quill) introduced on the side of the ligature nearest the heart. Subsequently sporadic reports of experiments with intravenous infusions appear in animals, for example the infusion of olive oil into dogs (Courten 1712), and later as a therapy for the fluid loss seen by patients suffering from cholera during the 19th century epidemics (Latta 1831). These trials were occasionally extremely successful and the consequent delight of their authors comes through their writings hundreds of years later. Unfortunately disappointment or disaster was a more frequent sequel to the trials. Even when the physiological basis for the infusion was sound (e.g. saline infusions to

cholera patients) almost all the other circumstances surrounding the giving of the infusion mitigated against its success. The new therapy was only tested on the moribund patients and then insufficient amounts of fluid might be given. The infusion itself was impure, unsterile and hypotonic resulting in immediate adverse events. A sceptical medical establishment (many of whose members believed further bleeding would benefit the cholera patient) was not convinced of the efficacy of any sort of intravenous therapy.

The closest 19th century equivalent of parenteral nutrition is the intravenous infusion of milk, suggested as a therapy for cholera. In a short trial it was dramatically successful in two out of three moribund patients, and may only have failed in the third patient because the infusion was insufficient. These promising experiments came to a close, however, when “Dr Bovell and myself then applied to the Corporation for a good cow, and a few articles indispensably necessary for the comfort and well-being of the patients; these were refused, and we thereupon sent in our resignations” (Hodder 1873).

3. Modern history

Clinically useful parenteral nutrition only became a possibility during the 20th century and a reality during its final quarter. The provision of safe and effective parenteral nutrition requires that basic concepts in physiology, nutrition, chemistry and materials science are correctly understood and integrated. The 18th and 19th centuries saw the discovery of many basic concepts in physiology and nutrition. Lind's experiments with citrus fruit in scurvy took place in 1747 and Lavoisier discovered the way food is oxidised and described calorimetry in the late 18th century. Advances in analytical chemistry during the

19th century showed that food was primarily C, N, H and O, and the relative quantities of these in carbohydrate, fat and protein (macronutrients) were calculated. The first half of the 20th century saw a refinement in our knowledge of the composition and metabolism of macronutrients and the identification of many micronutrients (vitamins and minerals). In the second half of the century, nutrition research has investigated the interaction between nutrients, the relationship between nutrition and disease and the provision of nutrition during disease. Experiments during the 1930s by Rose and Elman in particular, established the usefulness and practicality of amino acid infusions for the prevention and treatment of nutritional deficiencies (Elman 1937). At that time it became apparent that the amino acids were efficiently used only if sufficient non-protein calories were also supplied. Glucose was found to be of limited usefulness in this respect because of the high osmolality of solutions of the necessary concentration. Ethanol was used for some years as a source of calories in parenteral feeds, but has been abandoned because of toxicity and the development of a better alternative - lipid emulsions.

Lipid emulsions offer many advantages when included in the parenteral nutrition regimen. Rich in calories (9cal/g) they allow a high energy input in a relatively small volume, isotonic, solution. In addition they provide the only source of essential fatty acids. In common with ethanol, lipid infusions were attempted in animals in the same century as the circulation of the blood was discovered (Courten, 1678-9). Intravenous (and subcutaneous) administration of oil was attempted in the 19th and early 20th century but found to be neither practical nor useful. In the early part of the 20th century it was recognised that during normal nutrition, fat enters the blood stream from the thoracic duct as an

emulsion of chylomicrons. In the 1920s and 30s therefore experiments began with a variety of fat emulsions. In Sweden during the 1960s Wretlind refined the animal model in use (dogs) then by trial and error prepared an emulsion of soybean oil and egg yolk phospholipid which was later marketed as Intralipid (Hallberg et al 1966; Wretlind 1981). The particle size in Intralipid is similar to that of chylomicrons (0.5-1 μm). The infused lipid is metabolised in the same way as that absorbed from the diet. On entering the circulation the particles acquire apoprotein and are recognised and hydrolysed by lipoprotein lipase (Carpentier 1981).

The provision of safe total parenteral nutrition therefore became a realistic possibility. Although earlier reports of successful short term TPN exist (Helfrick & Abelson 1944), reports of TPN resulting in weight gain and growth in infants over a significant period of time appear from the late 1960s (Dudrick et al 1968; Wretlind 1992).

1.4 PARENTERAL NUTRITION IN NEONATOLOGY

1. Introduction

The development of effective intravenous nutrition has made the survival of sick and premature neonates possible where they might otherwise have simply starved to death. Estimates of the energy stores of a term infant suggest that survival would be possible for 30 days in the absence of nutrition. A small, premature infant might have stores sufficient for only 4 days. If 10% glucose is used to meet fluid requirements these figures are increased to 80 and 12 days respectively (Heird et al 1972).

2. Indications

Parenteral nutrition is used when normal enteral feeding is not possible or not tolerated. Almost all very low birthweight infants (those weighing less than 1500g) require intravenous nutrition during their first few days or weeks of life because of functional immaturity of their gastro-intestinal tract. In paediatric surgery, parenteral nutrition is used for infants with congenital gastro-intestinal anomalies such as atresias and gastroschisis. Parenteral nutrition may also be useful following abdominal surgery for any condition, when a post operative ileus can hinder the re-establishment of enteral feeding. Parenteral nutrition is valuable for infants with necrotizing enterocolitis. This condition is associated with prematurity and low birthweight and the avoidance of enteral feeding is part of its management. In all neonates the quantity of nutrients must be adequate for growth and development and not just survival (Hughes & Ducker 1981).

3. Route of administration

Parenteral nutrition may be administered into peripheral (arm or leg) or central (venae cavae) veins. The choice of route is usually dependent upon the expected or actual duration of the parenteral nutrition. Even when modern, isotonic solutions are used, cannulae in peripheral veins may only function for 2-3 days. The peripheral route is preferred for short term parenteral nutrition because it is initially more easily obtained and because central venous catheters are associated with their own significant complications. These include complications of insertion (arterial, pulmonary, lymphatic and neurological injury, air embolism and catheter malposition), infection and venous thrombosis (Turner 2000).

4. Composition

Carbohydrate, protein and fat are provided as glucose, synthetic amino acids and lipid emulsions respectively. Vitamins and trace elements are added to the infusion.

In the neonatal surgical patient it has been suggested that parenteral nutrition is commenced if a delay of greater than 4 days is anticipated before enteral feeding is tolerated (Lloyd & Pierro 1994). The precise regimen is tailored to suit the individual patient. Energy and protein needs are estimated on the basis of the patient's gestation, age, weight and clinical condition. Fluid requirements are also calculated from these parameters. At the Great Ormond Street Hospital for Children a computer program (Ascribe, ASC Computer Software Ltd.) calculates an appropriate regimen. The regimen is initially calculated on a daily basis and the quantities of glucose, amino acids, lipid and

fluid volume increased over a period of 5 days. When parenteral nutrition is provided by the central route the final daily neonatal prescription provides 18g/kg/day of carbohydrate, 3.36g/kg/day of amino acids and 4g/kg/day of fat in 150ml/kg/day of fluid.

5. *Metabolic complications of TPN*

It is necessary to monitor plasma electrolytes and nutritional indices to detect acute over or under-provision of sodium, potassium, calcium, phosphate or glucose. Over a longer period chronic deficiency syndromes may develop due to lack of essential fatty acids, zinc, copper, selenium, or vitamins.

6. *Types of lipid emulsion*

Triglycerides of long chain fatty acids (consisting of a carbon chain greater than 12 atoms in length) are the most abundant lipids in animal tissue and are the form of triglyceride present in Intralipid (Table 1.4.1 Stahl et al 1986).

Fatty acid	Content in Intralipid (%)
Palmitic acid C16:0	9
Stearic acid C18:0	3
Oleic acid C18:1 ω 9	26
Linoleic acid C18:2 ω 6	54
Linolenic acid C18:3 ω 3	8

Table 1.4.1 The fatty acid composition of Intralipid

Medium chain triglycerides possess carbon chains of 8 to 12 atoms in length. When given enterally they are directly absorbed into the blood, are more rapidly cleared from the blood and are more completely oxidised than long chain triglycerides (Metges & Wolfram 1991). Lipid emulsions have been developed in which long and medium chain triglycerides are provided as a 50:50 mixture (Bach & Babayan 1982). The predominant medium chain fatty acids in these solutions are caprylic (C8) and capric (C10) acids.

7. Complications of lipid emulsions

The development of a safe and effective source of fat was the last hurdle to be overcome in the development of a complete intravenous diet.

Complications that occurred with fat emulsions included hyperlipidaemia, febrile reactions, liver function abnormalities and fat deposition in the lung. Intralipid was synthesised by Wretling in Sweden in the mid 1960s, but such was the suspicion of fat emulsions following earlier disastrous experiences that in the USA the Food and Drug Administration did not approve its clinical use until 1976 (Macht 1980). Modern lipid emulsions are not free from undesirable side effects. Their use is associated with chronic lung disease (Cooke 1991), abnormal white cell function (Okada et al 1998), lipid deposition (Friedman et al 1978) and liver disease (Kelly 1998).

An increase in free radical production has been observed during the administration of TPN (Wispe et al 1985) and some of the above complications may be due to free radical mediated cell or tissue damage (Berger et al 1985).

1.5 FREE RADICALS AND PARENTERAL NUTRITION

1. Introduction and literature review

An increase in free radical production during TPN was first reported by Wispe et al in 1985. Studies were conducted both on infants receiving oral feeds or parenteral nutrition and on adult volunteers. In addition, animal studies were performed on newborn rabbits receiving either intravenous crystalloid or lipid solutions. The free radical indices used were expired ethane and pentane in the human studies and tissue and blood thiobarbituric reactive substances (TBARs, markers of free radical activity) in addition to ethane and pentane in the animal studies. He showed that the concentration of expired pentane was related to the duration of the lipid infusion and that it decreased within hours of stopping the infusion. Blood and tissue TBARs were increased in rabbits receiving lipid compared with controls. Van Gossum et al performed similar human studies in 1988, measuring breath pentane in adult volunteers and patients receiving home parenteral nutrition. The administration of a lipid emulsion caused a significant increase in expired pentane in both groups. Furthermore, the baseline pentane excretion (before lipid infusion) was higher in the home parenteral nutrition patients than the controls. Lipid peroxidation may have been increased because increased substrates have been provided, or as both Wispe and Van Gossum speculated, the lipid peroxidation products may already be present in the lipid emulsion before it is administered and cause the increase observed. Pitkanen et al in 1991 found that the expired pentane exceeded the amount administered by 3- to 4- fold in premature infants. Pitkanen concluded that lipid peroxidation

was occurring *in vivo* and may be responsible for some of the complications of Intralipid.

Helbock et al (1993) measured lipid hydroperoxide levels in 20% Intralipid using high-performance liquid chromatography with chemiluminescence detection.

Because exposure to light may result in further lipid peroxidation, measurements were made on fresh Intralipid samples and samples collected from the end of the intravenous tubing at the end of the infusion. Helbock speculated that the lipid hydroperoxide content of some lipid emulsions may represent a clinically significant risk to premature infants. There was no significant difference between levels in samples taken at the beginning and the end of the infusion.

Neuzil et al (1995) also investigated the effect of ambient and phototherapy light on 20% Intralipid. Triglyceride hydroperoxides were measured by HPLC with chemiluminescence detection. In contrast to Helbock, Neuzil found that hydroperoxide levels increased after 24 hours ambient or phototherapy light exposure compared with the baseline levels. He suggests that this phenomenon may result in the infusion of high concentrations of lipid peroxides being administered to premature infants and that this may represent a significant hazard.

Silvers et al (2001) demonstrated that lipid peroxidation could be decreased by using dark delivery tubing and also by adding multivitamins to the solution. Chessex et al (2001) agreed with the first of these findings but not with the second.

Since iron may act as a catalyst in the initiating steps of free radical formation, Lavoie and Chessex (1997) tested the effect of the addition of iron to TPN solutions. Hydroperoxide levels were measured in TPN solutions incubated with different iron additives. The iron additives tested were FeCl_2 , FeSO_4 , iron dextran and iron sorbitol. Incubations of the additives with TPN, with and without lipid, in the light at room temperature demonstrated that free iron induced free radical formation, whereas iron bound to dextran or sorbitol protected TPN solutions against peroxidation. On the basis of these studies Lavoie recommended the use of bound iron in PN for patients requiring supplementation.

Steger and Muhlebach (1997) measured in vitro oxidation of lipid emulsions in different all-in-one admixture bags. Peroxide levels in a long-chain triglyceride and a mixed medium and long-chain triglyceride emulsion were measured during storage at room temperature and daylight in bags made of ethylvinylacetate and polypropylene:polyamide. In contrast to storage in glass bottles, significant peroxidation was detected in both emulsions, with peroxide levels 150 times the control levels after storage in bags. The authors recommended that in order to prevent peroxidation, lipids in bags should be stored light-protected in a refrigerator.

Wanten et al (1999) measured free radical production by neutrophils (the 'respiratory burst') after lipid incubation. Neutrophils from healthy individuals were incubated in lipid emulsions in a physiological concentration containing long-chain triglycerides, mixed medium and long-chain triglycerides or structured triglycerides (synthesised triglycerides which may possess a combination of short, medium or long chain fatty acids on a single glycerol backbone). It was found that the LCT/MCT emulsion accelerated the neutrophil

respiratory burst whereas LCT (Intralipid) and structured lipid emulsions exerted no effect. Using different methodology, Heine et al (1999) also found that the MCT/LCT emulsion augmented the respiratory burst, and speculated that this effect may be due to a priming mechanism, since the emulsion itself did not cause the burst. Heine also found that LCT emulsions decreased the neutrophil respiratory burst following stimulation by *E. coli*, possibly due to decreased phagocytosis.

METHODS

2.1 FERROUS OXIDATION-XYLENOL ORANGE ASSAY FOR LIPID HYDROPEROXIDES

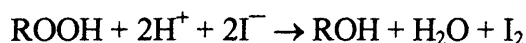
1. *Introduction*

As described previously, free radical attack upon polyunsaturated fatty acids begins with hydrogen abstraction from the methylene group adjacent to a carbon-carbon double bond. The resulting carbon centred radical undergoes molecular rearrangement to a diene-conjugate. Spontaneous reaction with oxygen results in a peroxy radical, which can itself abstract a hydrogen atom from an adjacent fatty acid. The peroxy radical forms a lipid hydroperoxide (LOOH) and further lipid peroxidation initiated (Figure 1.2.2). Lipid hydroperoxides are therefore one of the first relatively stable products resulting from free radical attack upon fatty acids.

2. *Measurement of lipid hydroperoxides*

Lipid hydroperoxides may be measured in several ways. Different hydroperoxides may be separated by high performance liquid chromatography (HPLC) or gas chromatography. Lipid peroxides may then be quantified by isoluminol chemiluminescence resulting from a reaction with LOOH derived free radicals (Brown & Kelly 1996) or by mass spectrometry (Halliwell & Gutteridge 1999 p.393).

The iodometric assay is based on the peroxide mediated oxidation of iodide to iodine:



In the presence of excess I^- tri-iodide (I_3^-) is produced which can be measured spectrophotometrically. Because iodide is oxidised by oxygen, the assay must be performed in an oxygen free environment (Gebricki & Guille 1989).

Cyclooxygenases catalyse the production of prostaglandins from arachidonic acid. The activity of the enzyme is stimulated by lipid peroxides and can be assayed by measuring oxygen uptake (Marshall et al 1985).

The ferrous oxidation xylenol orange (FOX) assay is based on the oxidation of iron (II) to iron (III) ions by peroxides. The iron (III) ion reacts with the dye xylenol orange to produce a colour change which can be measured spectrophotometrically. This reaction was used as the basis for an assay to determine micromolar quantities of H_2O_2 by Gupta (1973) and adapted by Jiang et al (1992) for measurement of lipid peroxides. I chose to use the FOX assay because of its relative simplicity and because the only special equipment required was the spectrophotometer.

3. Ferrous oxidation-xylenol orange assay

Under acidic conditions lipid hydroperoxides oxidise ferrous iron to ferric iron as follows:



In an acidic medium, ferric ions react with the dye xylenol orange (o-cresolsulfonephthalein-3'-3''-bis-(methyliminodiacetic acid sodium salt)) to form a complex which can be measured spectrophotometrically at 560nm.

Butylated hydroxytoluene, a lipid soluble antioxidant, inhibits further peroxide formation during the assay.

In order to correct for the presence of other peroxides (e.g. H_2O_2) triphenylphosphine (TPP), a reductant of lipid hydroperoxides is used. A simultaneous assay of the sample is performed with TPP added. Any reaction occurring when the samples have been incubated with TPP is due to non-lipid peroxides. The absorbance is subtracted from that obtained from the sample without TPP for calculation of the lipid peroxide concentration. (Nourooz-Zadeh et al 1994).

4. Materials

Xylenol orange, ammonium ferrous sulphate, H_2SO_4 , butylated hydroxytoluene, methanol, H_2O_2 , triphenylphosphine (Sigma Chemical Company, Poole, Dorset, UK).

Spectrophotometer (Uvikon 930 double beam spectrophotometer).

5. Preparation of reagents

Solution A consisted of 1mM xylenol orange, 2.5mM ammonium ferrous sulphate in 250mM H_2SO_4 . It was prepared by mixing 0.152g of xylenol orange and 0.196g of ammonium ferrous sulphate in 200ml of 250mM H_2SO_4 .

Solution B consisted of 4.4mM butylated hydroxytoluene in methanol. It was prepared by mixing 0.242g of BHT in 250ml of methanol.

A H_2O_2 standard solution was made in the range 0-100 μM

The 10 mM TPP solution was prepared by dissolving 0.131g in 50ml of methanol

6. Method

The working reagent was prepared by mixing reagents A & B in the ratio 1:9 (the “FOX” reagent). A standard curve was generated using the standard H₂O₂ solution, range 0-100μM.

90μl plasma was incubated with either 10μl TPP or 10μl of methanol at room temperature for 30 minutes. The sample was then mixed with 900μl FOX reagent in an Eppendorf tube and incubated for 30 minutes at room temperature. The samples were centrifuged at 12,000g for 5 minutes and the absorbance of the supernatant read at 560nm. The assay was performed in triplicate on each blood sample.

7. Calculation of the concentration of lipid peroxides

The concentration of lipid peroxides in the samples was calculated as follows:

$$[\text{LOOH}] = (A_M - A_T) \times 11.1/\epsilon$$

where A_M is the absorbance at 560nm by the sample incubated with methanol

A_T is the absorbance at 560nm by the sample incubated with TPP

11.1 is the dilution factor (90μl of sample in 1ml)

ϵ is the apparent molar extinction coefficient, obtained from the gradient of the standard curve (Figure 2.1.1).

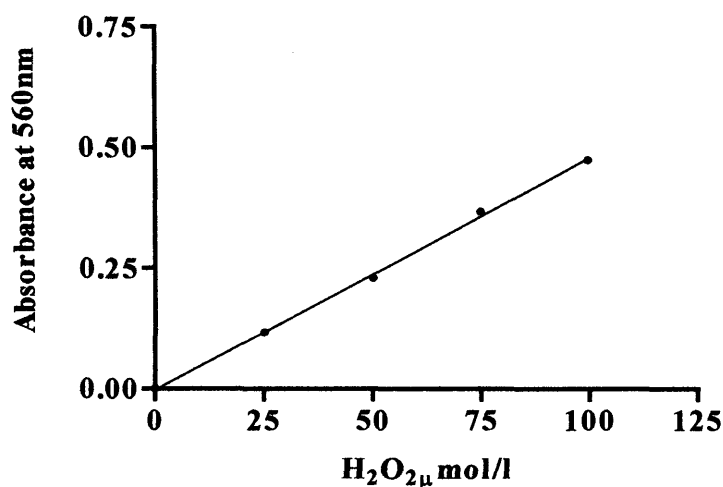


Figure 2.1.1 A standard curve for the FOX assay. Each point represents the mean of three measurements.

8. *EDTA interference with the assay*

In the initial clinical studies blood samples were collected into tubes which had EDTA as the anticoagulant. Attempts to perform the FOX assay for lipid peroxides on the samples failed. EDTA is a chelating agent for ferrous ions and may interfere with the assay.

Aim

To determine the effect of EDTA on the FOX assay.

Methods

The FOX assay was performed on 6 samples of a solution of 50μmol/l H₂O₂. EDTA was included in the assay in increasing concentrations from 10 to 500μmol/l. Each assay was performed in triplicate.

Results

The absorbance of the sample was reduced by the presence of EDTA in a concentration related manner (Figure 2.1.2). When the concentration of EDTA

was 100 $\mu\text{mol/l}$ the absorbance of the sample was reduced to less than half of the initial value; at 250 $\mu\text{mol/l}$ the absorbance is near to zero.

Conclusion

EDTA interferes with the FOX assay for lipid peroxides. As a result of this experiment blood samples were collected into EDTA tubes for the MDA assay (see below) and into heparinised tubes for the FOX assay.

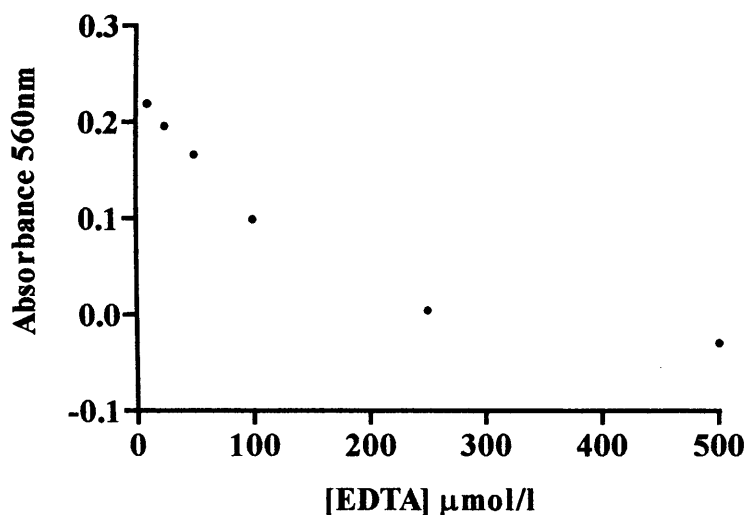


Figure 2.1.2 Graph showing the effect of increasing amounts of EDTA on the absorbance obtained in the FOX assay.

9. Intra-assay and inter-assay variation

A unit of plasma was obtained from the blood bank of the hospital and separated into 1ml aliquots which were stored at -70°C , to be used for quality control. To determine the intra-assay variation lipid peroxides were measured, in triplicate, in 15 of these samples. The mean concentration of lipid peroxides

was 2.17 μ mol/l, the standard deviation 0.06, and the coefficient of variation was 3%.

Whenever the assay was performed on patient samples an aliquot from the quality control plasma was analysed to obtain the variability of the results between different assay runs (the inter-assay variation). The mean concentration of lipid peroxides was 2.32 μ mol/l, the standard deviation 0.33, and the coefficient of variation 14% in six assay runs over a six month period.

Measurement of lipid peroxides in samples obtained from a given patient were performed in triplicate and were analysed in the same assay run, to eliminate potential differences caused by inter-assay variability.

2.2 1-METHYL-2-PHENYLINDOLE ASSAY FOR MALONDIALDEHYDE

1. Introduction

MDA ($C_3H_4O_2$) is a volatile, low molecular weight (formula weight 72) short chain weak acid (Janero 1990). It is one of the most widely quoted indices of free radical activity.

MDA is generated *in vivo* by the breakdown of lipid peroxides, but an additional source is the ingestion of MDA derived from polyunsaturated fatty acids in food (Draper & Hadley 1990). Metabolism and toxicity studies have been performed in cultured cells and in animals using orally administered and ^{14}C labelled MDA. These studies show that MDA is oxidised to CO_2 by aldehyde dehydrogenase and is also excreted unchanged in urine (Siu & Draper 1982).

MDA readily participates in condensation reactions with a range of chemicals to produce coloured products. Many of these reactions are used to assay MDA. One of the most commonly used assays utilizes the nucleophilic addition reaction with thiobarbituric acid - the TBA test. However MDA is not the only aldehyde to react with TBA and so the assay is said to measure thiobarbituric acid reacting substances ("TBARS"). A major drawback is that MDA may also be produced during the performance of the test itself, when the sample is heated with acid, causing decomposition of lipid peroxides. A range of other tests are also based on the reaction of MDA with nucleophiles (e.g. anthrone, *p*-aminobenzoic acid, quinine), to produce fluorescent or light

absorbing products (Janero 1990). MDA may also be measured directly by HPLC and UV spectrophotometry (Esterbauer & Cheeseman 1990).

For these studies a colorimetric assay was chosen in which MDA reacts with 1-methyl-2-phenylindole, a chromogenic reagent which yields a stable chromophore with a maximal absorbance at 586nm (Figure 2.2.1). This assay has been validated against HPLC measurement and shown to be specific for MDA (Diaz et al 1998).

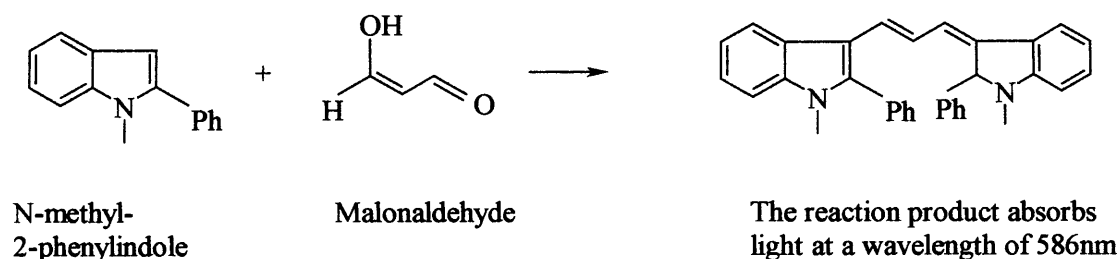


Figure 2.2.1 The MDA assay. MDA reacts with 1-methyl-2-phenylindole to yield a stable chromophore with a maximal absorbance at 586nm

2. 1-methyl-2-phenylindole assay for plasma MDA

Plasma MDA was measured by heating samples under acidic conditions with 1-methyl-2-phenylindole to yield a stable chromophore with a maximal absorbance at 586nm.

3. Materials

1-methyl-2-phenylindole, acetonitrile, 1,1,3,3-tetramethoxypropane (standard), (supplied as a kit by R&D Systems, Europe, Ltd.).

Hydrochloric acid 37%, methanol (Sigma Chemical Company, Poole, Dorset, UK).

Spectrophotometer (Uvikon 930 double beam spectrophotometer).

4. Preparation of reagents

A solution of 10.3mM 1-methyl-2-phenylindole in acetonitrile was prepared by dissolving 0.213g of solute in 100ml acetonitrile. This stock solution was diluted in methanol in a ratio of 4 to 1 to make solution A.

An aqueous solution of standard 1,1,3,3, tetramethoxypropene in 20mM Tris HCl buffer was diluted 100-fold in de-ionised water.

5. Methods

To 650µl of solution A, 200µl of plasma sample and 150µl of 37% HCl were added. The resulting mixture was vortexed and incubated at 45°C for 60 minutes. At the end of the incubation, the sample was cooled on ice and centrifuged for 10 minutes at 12,000g. The absorbance of the supernatant was measured at 586nm.

The standard 1,1,3,3-tetramethoxypropane in water was used to construct a standard curve in the range 0-10µmol/l.

6. Calculation of the concentration of MDA

The concentration of MDA in the samples was calculated as follows:

$$[\text{MDA}] = (A - A_0) \times 5 / \epsilon$$

where A is the absorbance at 586nm by the sample

A_0 is the absorbance at 586nm by the blank (water)

5 is the dilution factor (200 μ l of sample in 1ml)

ϵ is the apparent molar extinction coefficient, obtained from the standard curve

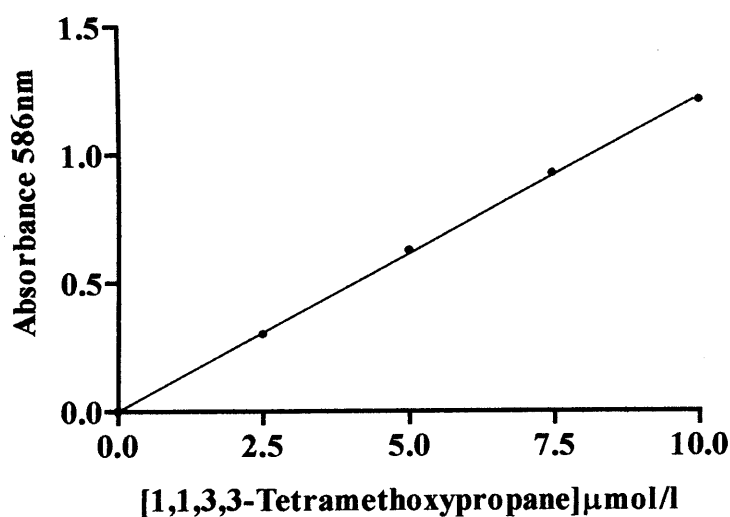


Figure 2.2.2 A standard curve for the MDA assay. Each point represents the mean of three measurements.

7. Inter and intra-assay variation

A unit of plasma was obtained from the blood bank of the hospital and separated into 1ml aliquots which were stored at -70°C. MDA was measured in 15 of these samples and the intra-assay coefficient of variation for the MDA assay obtained from the results. The mean concentration of MDA was 4.85µmol/l, the standard deviation 0.16, and the coefficient of variation 3%.

Whenever the MDA assay was performed, one of the aliquots from the quality control plasma was analysed to obtain the inter-assay coefficient of variation. The mean concentration of MDA was 5.61µmol/l, the standard deviation was 0.79, and the coefficient of variation 14% over 30 assay runs during a six month period.

As with the lipid peroxide assay, the samples obtained from a given patient were always analysed in triplicate and in the same assay run, eliminating potential differences caused by intra-assay and inter-assay variability.

2.3 PENTANE

1. Introduction

Pentane is a 5-carbon gas which may be formed by the decomposition of peroxides of ω -6 fatty acids (Figure 1.2.2). Measurement of expired breath pentane therefore offers a non-invasive method of assessing in vivo lipid peroxidation. In vitro studies have shown that the pentane formation correlates well with other indices of free radical activity and is possibly the most sensitive test of lipid peroxidation (Kneepkens et al 1994). Expired pentane has been used in many studies of TPN associated free radical activity (Wispe et al 1985; Van Gossum et al 1988; Pitkanen et al 1989; Marshall & Roberts 1990) and has been correlated with increased mortality in sick neonates (Nycyk et al 1998).

The infants studied breathed hydrocarbon free air to avoid contamination by atmospheric hydrocarbons. The materials of the breath collection apparatus were free from rubber and grease. The breath samples were analysed in the Department of Neonatology at Liverpool Women's Hospital. Previous studies showed that pentane levels were stable in the collection bags for up to 48 hours (Drury et al 1997).

2. Materials

Hydrocarbon free air (<0.1ppm), BOC Special Gases, Guildford, Surrey

Cis-2-pentene (10ppm), BOC Special Gases, Guildford, Surrey

Tedlar bags, Supelco Inc., Bellafonte, PA, USA

Carbotrap, Supelco UK, Poole, Dorset, UK

Neoprine face-mask

One way valves

3. Method of breath collection

Infants were placed in a perspex canopy ventilated with hydrocarbon free air for a 10 minute washout period.

Timed breath collections were made over 2-3 minutes into Tedlar bags via a face-mask and a one way valve circuit (Figure 2.3.1 and 2.3.2). Measurements were performed in triplicate.

200µl of 10ppm cis-2-pentene were added to each bag as an internal standard.

The filled bags were packed in boxes and dispatched to Liverpool Women's Hospital by overnight postal delivery.

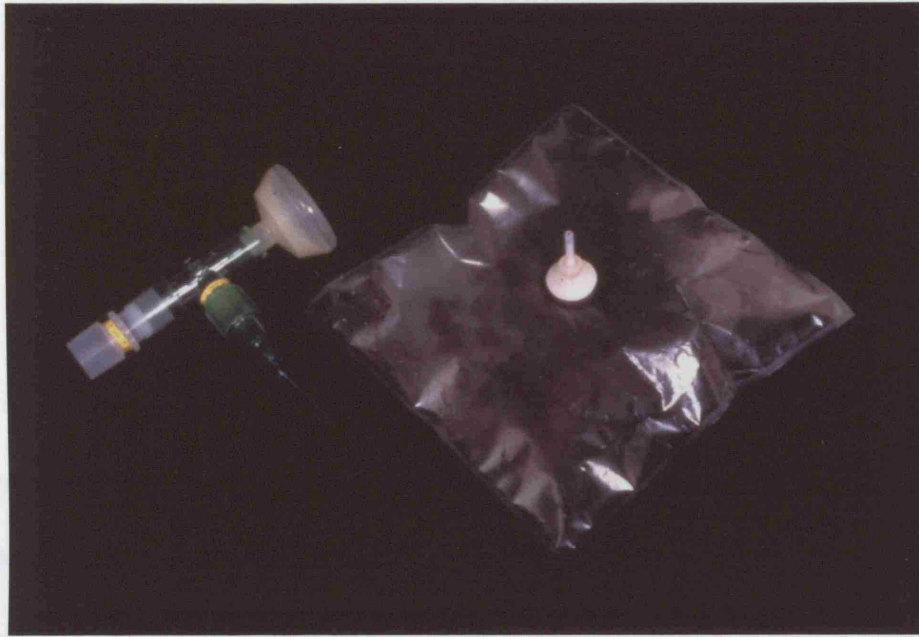


Figure 2.3.1 Breath collection equipment



Figure 2.3.2 Breath collection from an infant in the study

4. Method of pentane analysis

Expired breath samples were transferred by vacuum from the Tedlar bags onto sample tubes packed with Carbotrap, a graphitised carbon resin which adsorbs the hydrocarbons from the sample.

The samples were desorbed from the sample tubes at 300°C, cryofocussed in a cold trap at -180°C, and injected from the cold trap into the gas-chromatograph at 200°C. Separation was carried out using a Chrompack 9001 GC with an Al₂O₃/KCl PLOT column from 50 to 180°C, and detected using a flame ionisation detector at 180°C.

A calibration mix of ethane, ethene, ethyne, isopentane, pentane and cis-2-pentene in air was used for identification and calculation of concentrations. Analyses were carried out by J.A. Drury. Sample chromatograms are shown in Figure 2.3.3a and b.

Breath pentane measurements were only available for Study 5.

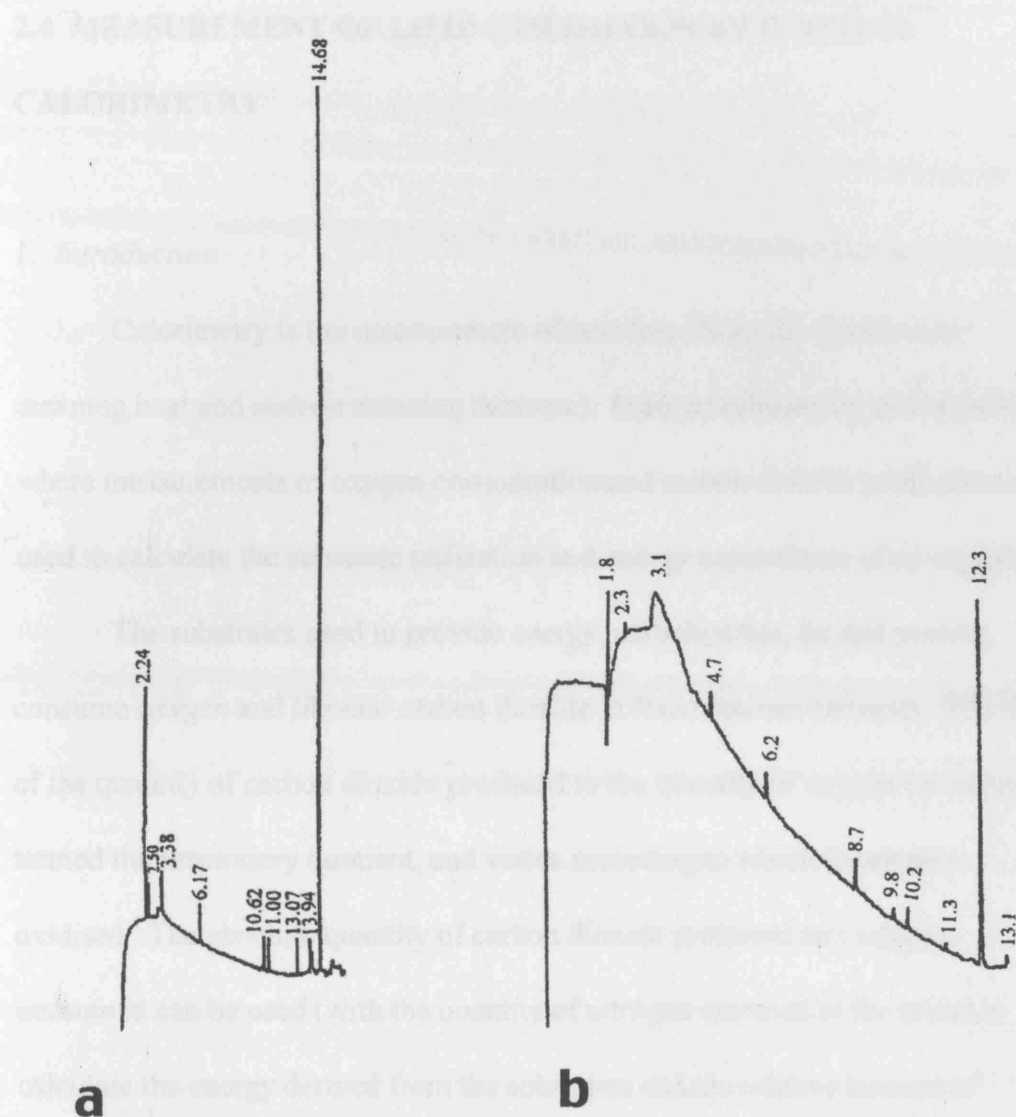


Figure 2.3.3 Sample chromatograms for pentane analysis

a) Separation of standards: Isoprene (14.68 min), ethane (2.50 min), ethene (3.38 min), ethyne (6.17 min), isopentane (10.62 min), n-pentane (11.00 min), cis-2-pentene (13.07 min).

b) Normal sample Pentane, 10.2 min; cis-2-pentene, 12.3 min.

2.4 MEASUREMENT OF LIPID UTILIZATION BY INDIRECT CALORIMETRY

1. *Introduction*

Calorimetry is the measurement of heat loss (from the Greek *calor* meaning heat and *metron* meaning measure). Indirect calorimetry is a technique where measurements of oxygen consumption and carbon dioxide production are used to calculate the substrate utilization and energy expenditure of an organism.

The substrates used to provide energy, carbohydrate, fat and protein, consume oxygen and liberate carbon dioxide in fixed, known amounts. The ratio of the quantity of carbon dioxide produced to the quantity of oxygen consumed is termed the respiratory quotient, and varies according to which substrate is oxidised. The absolute quantity of carbon dioxide produced and oxygen consumed can be used (with the quantity of nitrogen excreted in the urine) to calculate the energy derived from the substrates and the relative amount of carbohydrate, fat and protein utilized.

2. *History of indirect calorimetry (reviewed by MacFie 1982 and Webb 1991)*

John Mayow, an English chemist, observed that when a candle and an animal were enclosed in an airtight container the candle was extinguished at the same time as the animal died (1674). Mayow recognised that something was present in the air which was necessary to maintain life. He termed this "spiritus nitroaereus". In the 18th century Joseph Priestley isolated this fraction of the air, calling it "dephlogisticated air". Working at the end of the 18th century, Antoine Lavoisier recognised this substance as an element and termed it oxygen.

Lavoisier studied both nutrition and combustion of foodstuffs. From his measurements of oxygen consumption and carbon dioxide production in living organisms, he concluded that the major part of animal heat originated from the combustion of organic substrates with oxygen in the body. This is the principle underlying the technique of indirect calorimetry to determine an organism's energy expenditure. Some of his work was performed on his ice calorimeter, developed with Laplace. Heat loss from an experimental animal was determined from the quantity of water obtained from melted ice in a chamber surrounding the animal.

The first apparatus for measurement of respiratory gas exchange in animals was described by Regnault and Riesel in 1849. A "closed circuit" was used to supply oxygen and remove carbon dioxide. In 1862 Pettenkofer and Voit, funded by King Maximilian II of Bavaria, built a respiratory chamber large enough for human experiments. Rubner, a pupil of Voit, built a respiration chamber which also measured heat loss and was therefore able to demonstrate the relationship between indirect and direct calorimetry. In America, Atwater (1844-1907) and Rosa used a combined direct and indirect calorimeter to study normal metabolism. Their data has been the basis of subsequent metabolic studies. All these experiments were conducted in a closed circuit - i.e. the same air circulated around the chamber. This limited the duration of the studies due to falling oxygen levels. In the 1890s Zuntz developed the first "open circuit" calorimeter in which a valve system separated inspired and expired air, allowing analysis of the latter and an unlimited supply of the former. The original calorimeters were chambers in which an animal could live, or rooms equipped with a bed and chair for human studies. In 1930 Benedict described a "helmet"

for studies of "gaseous metabolism" and in 1963 Kinney designed a plastic hood for collecting expired air.

Open circuit indirect calorimetry using a perspex hood is now widely used for studies of energy expenditure in the laboratory and hospital. Calorimetry has also benefited from advances in instrumentation and computers, allowing the production of much smaller machines than in the past. The resulting portability has greatly facilitated the conduct of metabolic studies on critically ill patients and on infants and neonates.

3. Calorimeter

Datex Deltatrac Metabolic Monitor, Instrumentarium Corp., Helsinki, Finland (Figure 2.4.1). The calorimeter may be used at relatively low flow rates and has been validated for use in metabolic studies in preterm and term neonates (Shortland et al 1992).

4. Method of indirect calorimetry

In the present study a canopy was positioned around the patient's head and the expired air was drawn from the hood at a constant flow rate of 3l/min (Figure 2.4.2). Inspired and expired gases were analysed by a paramagnetic O₂ analyser and an infrared CO₂ analyser. The patient's whole-body O₂ consumption ($\dot{V}O_2$) and CO₂ production ($\dot{V}CO_2$) were calculated from the difference in O₂ and CO₂ concentrations between inspired and expired air and the air flow rate through the calorimeter. During the study the infant's physical activity was monitored minute by minute to separate resting from crying and/or

total body movements. Studies were performed for 1 to 2 hours at the same time each day.



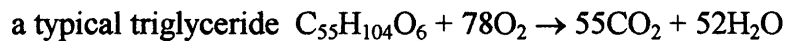
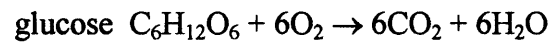
Figure 2.4.1 The Datex Deltatrac Calorimeter



Figure 2.4.2 An infant in the study undergoing indirect calorimetry

5. Calculation of lipid utilization and energy expenditure

Lipid utilization and energy expenditure were calculated from the $\dot{V}O_2$ and $\dot{V}CO_2$ according to the principles of indirect calorimetry. These are based on the stoichiometry of substrate oxidation reactions:



When one molecule of glucose is oxidised, the relationship between the amount of CO_2 produced and O_2 consumed is 1.0. This relationship, the respiratory quotient, for a typical molecule of fat is 0.7. The relative fractions of glucose and lipid oxidised may be calculated by using the known respiratory quotients and the actual respiratory quotients measured by calorimetry (Ferranini 1988). For the purposes of these calculations it is assumed that the oxidation of the metabolic fuels follows a standard, known stoichiometry. Also that the study is sufficiently long that short term fluctuations in body pools may be ignored (Swyer 1991). There is virtually no body store of oxygen, therefore the O_2 consumption as measured using expired air closely reflects the actual quantity of oxygen used. However endogenously produced CO_2 enters a large bicarbonate pool which may permit alterations to occur in its rate of excretion.

Previous nitrogen balance studies (3-day urine collection) on 21 surgical infants with similar clinical conditions and receiving TPN have demonstrated a linear relationship between timed urinary nitrogen excretion and caloric intake (Jones and Pierro, unpublished data). The urinary nitrogen excretion in this study was calculated from this relationship. By applying the equations of indirect calorimetry (below) to our measurements of $\dot{V}O_2$, $\dot{V}CO_2$ and urinary

nitrogen excretion, the non-protein respiratory quotient and the net lipid utilization were calculated.

The following abbreviations and constants are used in the equations:

CHO = Carbohydrate

RQ = respiratory quotient

REE = resting energy expenditure

fr- = fraction of substrate utilization from-

6.25 = grams of protein equivalent to 1 g of nitrogen

9.25 = Caloric value (kcal/g) of lipid

3.75 = Caloric value (kcal/g) of carbohydrate

5.65 = Caloric value (kcal/g) of protein

5.047 = Oxygen (kcal/l) produced by carbohydrate oxidation

4.686 = Oxygen (kcal/l) produced by lipid oxidation

0.707 = RQ of lipid oxidation

1.000 = RQ of carbohydrate oxidation

0.293 = difference in RQ between that of carbohydrate and
lipid oxidation

0.800 = RQ of protein oxidation

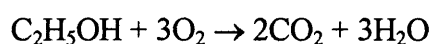
0.9663 = Oxygen (l) required to oxidise 1 g of protein

0.7739 = CO₂ (l) produced by oxidation of 1 g of protein

- (1) protein oxidation (g/kg/day) = urinary nitrogen (g/kg/day) x 6.25
- (2) non-protein $\dot{V}O_2$ (l/kg/day) =
 $\dot{V}O_2$ (l/kg/day) - (protein oxidation x 0.9663)
- (3) non-protein $\dot{V}CO_2$ (l/kg/day) =
 $\dot{V}CO_2$ (l/kg/day) - (protein oxidation x 0.7739)
- (4) non-protein RQ = non-protein $\dot{V}CO_2$ / non-protein $\dot{V}O_2$
- (5) fr-CHO = (non protein RQ - 0.707) / 0.293
- (6) fr-fat = (1 - non-protein RQ) / 0.293
- (7) net CHO oxidation (g/kg/day) =
(fr-CHO x non protein $\dot{V}O_2$ x 5.047) / 3.75
- (8) net fat oxidation (g/kg/day) = (fr-fat x non protein $\dot{V}O_2$ x 4.686) / 9.25
- (9) non-protein REE (kcal/kg/day) =
(CHO oxidation x 3.75) + (lipid oxidation x 9.25)
- (10) REE (kcal/kg/day) = non-protein REE + (protein oxidation x 5.65)

6. Calibration and variation

The calorimeter was calibrated before each use with a mixture of 5% CO₂/95% O₂, from a gas cylinder stored on the machine. The accuracy of the respiratory quotient and energy expenditure measurement was regularly checked by burning a known volume (5ml) of 99% ethanol in a glass jar connected to the calorimeter. Ethanol consumes O₂ and produces CO₂ in the ratio 3 to 2:



The calibration procedure was performed on 14 occasions over a 14 month period. During the test the mean (SD) $\dot{V}O_2$ was 299.5 ml/min (18.06ml/min), $\dot{V}CO_2$ was 201.8ml/min (10.56ml/min), RQ 0.67 (0.01) and

energy expenditure 1989kcal/day (108.2kcal/day). The coefficient of variation for the respiratory quotient was 1.7% and for energy expenditure 5.4%.

2.5 THE PAEDIATRIC RISK OF MORTALITY (PRISM) SCORE

1. Introduction

The measurement of illness severity is useful to assist in comparing outcomes between treatments or institutions, the allocation of resources, assisting clinical decision making and for assessing prognosis.

Severity scores may be applied to patients with a particular diagnosis, or based on risk factors, therapeutic interventions or physiological indices (Richardson & Tarnow-Mordi 1994).

Scores for patients with a specific diagnosis are familiar in all disciplines. The Glasgow coma score after head injury and the tumour, nodes, metastases (TNM) grading of malignancies are commonly used measures. Such scores fulfill the criteria for an "ideal" illness severity scale, being simple, derived from readily available information, reliable and valid.

Scales based on risk factors are similarly close to ideal scoring systems. An example is the American Society of Anesthesiologists grading of fitness for anaesthesia.

The rapid development of intensive care, the complexity of the patients' disorders and the increasing cost and sophistication of interventions renders a scoring system particularly useful in this setting. However, scores developed for intensive care use have not been so close to the "ideal" system, therefore no one score has become universally applied.

The Therapeutic Intervention Scoring System (TISS) was described by Cullen in 1974. It was one of the first scoring systems developed for use in

intensive care and is based on the number and intensity of therapies used for a particular patient. A neonatal version has also been developed (Gray et al 1992).

Severity scores based on physiological indices are appropriate for an intensive therapy setting because the required data is easily defined and collected and the score is independent of the diagnosis. The first of these was the Acute Physiology and Chronic Health Evaluation (APACHE) score. Points are allocated according to age, physiological indices and chronic illnesses to derive the score during the first 24 hours after admission.

The Physiologic Stability Index (PSI) was derived from the APACHE system for use in paediatrics (Pollack et al 1987). Thirty four variables were measured on admission and coded into 75 variable ranges. Derangements were assigned 1, 3 or 5 points depending on their degree. Because paediatric physiology is not uniform throughout childhood, age adjustment is necessary for some variables. This complex index was simplified to the Paediatric Risk of Mortality (PRISM) score (Pollack et al 1988), with 14 variables.

In our unit, the data required to calculate the PRISM score is collected by the nursing staff and entered into a computer at the bedside. The score was calculated by an add on computer program and was available for each child on a daily basis.

2. Materials and method

The variables were recorded by nursing staff into a bedside electronic chart (HP CareVue 9000, Hewlett-Packard, Palo Alto, USA) and the PRISM score derived using an add-on computer program (Cedars Sinai Medical Center, NY, USA).

3. Calculation of the PRISM score

The information used to calculate the PRISM score is detailed in the table below. The systolic blood pressure (BP), heart rate and respiratory rate were adjusted for age. The variables used to calculate the score were only those which are obtained for clinical care, no variable was measured solely to produce the score. The score was not adjusted for diagnosis.

Variable	Age ranges		Score
Systolic BP (mm Hg)	Infants	Children	
	130-160	150-200	2
	55-65	65-75	2
	>160	>200	6
	40-54	50-64	6
	<40	<50	7
Diastolic BP (mm Hg)	>110		6
Heart rate (beats/minute)	>160	>150	4
	<90	<80	4
Respiratory rate (breaths/minute)	61-90	51-70	1
	>90	>70	5
PaO ₂ /FiO ₂	200-300		2
	<200		3

3. Calculation of the PRISM score continued

Variable		Score
PaCO ₂ (torr)	51-65	1
	>65	5
Glasgow coma score	<8	6
Pupillary reaction	unequal or dilated	4
	fixed dilated	10
PT/PTT	1.5 x control	2
Total bilirubin	>3.5 if age >1mnth	6
Potassium (mEq/l)	3.0-3.5	1
	6.5-7.5	1
	<3.0	5
	>7.5	5
Calcium (mg/dl)	7.0-8.0	2
	12.0-15.0	2
	<7.0	6
	>15	6
Glucose (mg/dl)	40-60	4
	250-400	4
	<40	8
	>400	4
Bicarbonate (mEq/l)	<16 or >32	3

The risk of mortality (r) may be calculated as follows:

$$r = (0.27 \times \text{PRISM score}) - (0.005 \times \text{age (months)}) \\ - (0.433 \times \text{operative status}) - 4.78$$

where operative status = 0 if no operation has been performed

operative status = 1 if the patient has had an operation

2.6 MEASUREMENT OF PLASMA CYTOKINES

1. Introduction

Cytokines are a structurally diverse group of proteins responsible for initiating and controlling the inflammatory response. TNF- α and IL-6 are two major pro-inflammatory cytokines. They are released during the few hours following infection by activated monocytes, macrophages and lymphocytes. They act at both the site of an infection to activate leucocytes and systemically in production of fever and promoting acute phase protein synthesis. Cytokine levels have been correlated with clinical severity of illness in infections (Bhutta et al 1997; van Deuren et al 1995) and with the PRISM score in children with septic shock (Hazelzet et al 1994). It has been shown that TNF enhances superoxide production by neutrophils (Jersmann et al 1998) and that TNF mediated cytotoxicity is associated with free radical generation. Contrasting findings suggest that TNF has free radical scavenging properties in cancer cells (Matsubara et al 1997).

Cytokine levels on small plasma samples can be detected using enzyme linked immunosorbent assay methods (ELISA).

The cytokines TNF- α and IL-6 were measured by a double antibody, two-site (sandwich) assay. In this type of assay a “capture” antibody is attached to the microplate plate and binds the cytokine to be measured. A second “detection” biotinylated antibody is conjugated to an enzyme and binds to the analyte captured by the first antibody. Horseradish peroxidase conjugated streptavidin binds onto the biotinylated side of the sandwich. After removal of

non-bound horseradish peroxidase conjugate by washing, a substrate solution is added to the wells. A coloured product is formed in proportion to the amount of cytokine present in the sample or standard. After the reaction has been terminated by the addition of a stop solution, absorbance is measured in a microtiter plate reader. From the absorbance of samples and those of a standard curve, the concentration of the cytokine can be determined by interpolation with the standard curve.

2. Materials

Wash buffer: 0.05% Tween 20 in phosphate buffered saline

Blocking solution: 1%BSA, 5% sucrose, 0.05% NaN₃ in phosphate buffered saline

Diluent: 0.1% BSA, 0.05% Tween 20 in Tris buffered saline

0.05 M Phosphate Citrate buffer

Substrate solution: (1 tablet tetramethylbenzidine, 2 µl H₂O₂/ 10 ml phosphate-citrate buffer)

Stop Solution: 4N H₂SO₄

Capture antibody solution: 4 µg/ml

Detection antibody solution: 200 ng/ml (IL-10: 400 ng/ml)

Antibody pairs (R&D Systems, Europe, Ltd):

Human TNF-α

Capture antibody	MAB 610
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Detection antibody	BAF 210
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Human IL-6

Capture antibody MAB 206

Detection antibody BAF 206

3. Method

Each well in the microtitre plate was coated with 75 µl capture antibody solution. The plate was sealed and incubated overnight at room temperature. The plate was washed with the wash buffer before blocking with 200µl well blocking solution at room temperature for at least 1 hour. Following further washing, 75µl of standard or sample was added. The plate was covered and incubated for 2 hours at room temperature. Following removal of the antibody and thorough washing 100µl of streptavidin horseradish peroxidase diluted 1:1000 in diluent was added to each well. The plate was covered and incubated for 20 minutes at room temperature.

The plate was washed with buffer and 100 µl/well of substrate solution was added. The plate was covered and incubated for 20 minutes at room temperature in the dark. The reaction was then terminated with addition of 50 µl of the stop solution to each well.

The optical density was read within 30 minutes at 450 nm.

2.7 CLINICAL STUDIES

1. Patient selection

The metabolic studies were performed on surgical infants receiving TPN because of postoperative gut dysfunction or in the recovery phase from necrotizing enterocolitis. The infants were free from sepsis, received no enteral feeds and were clinically stable over the course of the study. During the study each patient received continuous TPN. The intravenous fluid intake varied from 100 to 180 ml/kg/day according to the patient's requirement. Patients requiring mechanical ventilation were included in the studies provided that their clinical condition was stable and ventilator parameters kept constant. Patients were withdrawn if, during the study, their TPN was interrupted (e.g. due to central line blockage) or if a major clinical problem developed (e.g. sepsis).

2. Recruitment and consent

Parents were approached regarding their child's participation in the study when his or her clinical condition was stable and when TPN therapy was being established. The reasons for the study, its risks and benefits were explained and an information sheet provided. A repeat appointment with the parents was arranged during the subsequent 24 hours and written consent obtained for participation if the parents agreed. The assistance of an interpreter was available when English was not the parent's first language.

3. TPN prescription

Carbohydrate, amino acids, electrolytes, vitamins, and trace elements were provided as Vamin 9® solution, Pharmacia & Upjohn, Milton Keynes, Bucks, U.K. The lipid infusion was Intralipid® 20%, Pharmacia & Upjohn, Milton Keynes, Bucks, U.K. When the medium chain triglyceride was investigated, the lipid used was Lipofundin® 20%, B.Braun, Melsungen, Germany.

Precision infusion pumps (IVAC Corporation, San Diego, CA, USA) were used for the TPN infusion in all patients.

The TPN prescription was written on a daily basis to provide the nutrients according to the patient's weight. A computer program, Ascribe, ASC Computer Software Ltd. calculated the actual amounts of each component which were mixed on the day of use for an individual patient. When parenteral nutrition was provided by the central route the final daily neonatal prescription provided 18g/kg/day of carbohydrate, 3.36g/kg/day of amino acids and 4g/kg/day of fat in 150ml/kg/day of fluid.

4. Blood sampling

Blood samples for lipid peroxide and MDA analysis were obtained at the same time each day, either by a heel prick or from the central venous infusion line. The same method was used on each occasion in any individual patient. The samples were obtained at the same time as blood required for clinical tests to minimise distress to the patient.

The samples were centrifuged at 2,500rpm for 5 minutes, the plasma separated and stored under nitrogen at -70°C until analysed.

5. Ethical approval

The studies were approved by the ethics committee of Great Ormond Street Hospital for Children.

2.8 STATISTICAL TESTS

Normally distributed data are presented as the mean and the range.

Non-normally distributed data as the median and range.

Student's t test was used for paired and unpaired parametric data.

Regression analyses were used to determine the relationship between independent variables.

Unpaired non-parametric data were tested using the Mann-Whitney test.

Spearman's rank correlation was used to test for a relationship between variables.

The Kruskal-Wallis test (a non parametric one way analysis of variance test) was used to compare three or more unpaired groups. The post hoc analysis used Dunn's multiple comparison test to determine differences between groups.

EXPERIMENTS

3.1 DOES LIPID PEROXIDATION OCCUR IN LIPID EMULSIONS?

1. Introduction

In view of the potential for lipid emulsions to cause free radical mediated cell damage, I wished to investigate whether free radical formation during TPN therapy could be reduced. In my initial studies I wished to confirm the presence of ~~presence of~~ increased indices of free radical activity in the lipid solutions themselves and in plasma samples from patients receiving TPN.

The presence of lipid peroxides and their degradation products in lipid emulsions has been demonstrated by many groups investigating free radical formation in patients receiving TPN (Helbock et al 1993; Neuzil et al 1995; Lavoie et al 1997; Silvers et al 2001). These chemicals may themselves be a source of the toxicity of lipid emulsions (Helbock et al 1993). In addition, the presence these products in vivo is interpreted as evidence of free radical activity. It is possible that the lipid peroxides measured in vivo may simply be the result of their being infused in the TPN regimen.

Some of the studies referred to above give the concentrations of lipid peroxides in the lipid emulsions. However the quantities vary, sometimes widely. Therefore I decided to measure the concentration of MDA and of lipid peroxides in the intravenous lipid being administered to the patients in the following studies using my own assays.

2. Aim

To measure the quantities of MDA and lipid peroxides in lipid infusions during their administration.

3. Materials and methods

Six samples of Intralipid 20% were obtained at the bedside from the intravenous giving sets of six infants on TPN. The samples were stored at 4°C and analysed for lipid peroxide and MDA levels on the following day.

The samples were analysed in triplicate.

Because Intralipid is an opaque solution each assay was performed against a blank containing Intralipid and all the reagents except the 1-methyl-2-phenylindole in the MDA assay. The lipid peroxide assay routinely incorporates a blank containing the sample in all assays.

4. Results

The results are shown in Table 3.1.1. The mean concentration of MDA was 8.69µmol/l (range 5.42 - 12.69µmol/l) and for lipid peroxides 19.25µmol/l (range 11.47 - 30.75µmol/l).

MDA $\mu\text{mol/l}$	Lipid peroxides $\mu\text{mol/l}$
12.6	23.3
9.9	16.5
5.7	11.5
5.8	17.9
5.4	15.6
12.7	30.8

Table 3.1.1 MDA and LOOH in six samples of Intralipid 20%.

There was a significant positive correlation between MDA and lipid peroxide concentrations, $r=0.8$, $p<0.05$ (Figure 3.1.1).

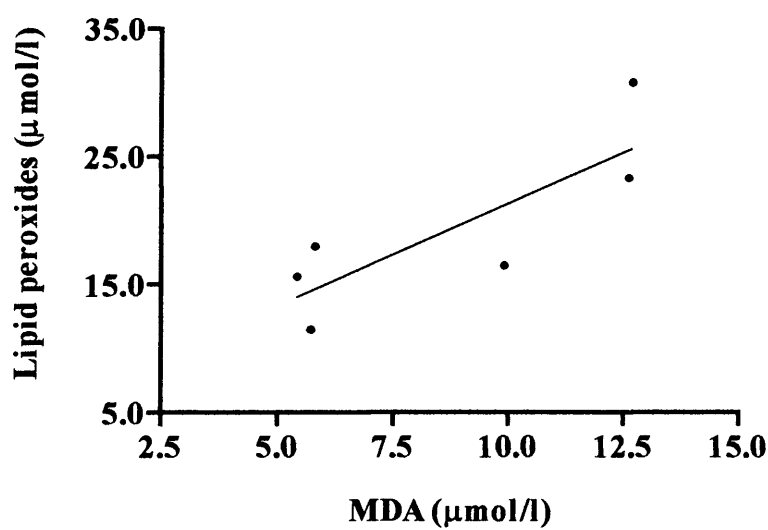


Figure 3.1.1 Correlation between MDA and LOOH levels in Intralipid 20%.

5. Discussion

Experiment 1 confirmed the presence of lipid peroxides and MDA in lipid emulsions. These are the products of free radical mediated degradation of unsaturated fatty acids and their presence implies free radical activity in the solution. The formation of free radicals in the infusate may occur because of exposure to heat, light or oxygen, all of which initiate lipid peroxidation via free radical intermediates (Neuzil et al 1995; Steger & Muhlebach 1997). The material of the bag containing the lipid emulsion has also been implicated in causing lipid peroxidation. The initial formation of free radicals begins a chain reaction in which lipid peroxide radicals react with adjacent unsaturated fatty acids to produce more of the same.

If free radical mediated chain reactions are occurring in the solution as it hangs by the bedside, it is possible that free radicals are being infused directly into the circulation of newborn infants receiving lipid emulsions. Infused lipids are carried in particles similar to chylomicrons (Stahl et al 1986). The carbon chain of the lipid is in the core of the particle, therefore continuing free radical activity involving the side chain may be expected to be restricted to this micro-environment. Fatty acids are liberated from the particle by endothelial lipoprotein lipase and are taken up into tissues or are released into the circulation where they bind to albumin. If at this point, the fatty acid is engaged in a free radical reaction, it is conceivable that “bystander” lipid molecules (such as those of the endothelial cell membrane) may become involved in the reaction.

Lipid peroxides and their degradation products are toxic. In vitro studies have shown that lipid peroxides are toxic to fibroblasts and vascular endothelial cells, probably due to their properties as initiators of free radical reactions. Their

concentrations in an intravenous lipid emulsion are of interest because they may reach a level that might cause adverse effects. Elimination of lipid peroxides occurs mainly by degradation to toxic products including formaldehyde, acetaldehyde, acetone, propanal, butanal, and MDA (Esterbauer 1993). Therefore the toxic effect of a given concentration of lipid peroxides may be more than that which would be expected from the lipid peroxides alone.

MDA is mutagenic (Basu & Marnett 1983) and reacts with the amino groups of protein molecules to damage protein structure (Halliwell & Gutteridge 1999, p.301). MDA is eliminated by:

- i) oxidation to carbon dioxide
- ii) reduction of the aldehyde to an alcohol
- iii) renal excretion (Draper & Hadley 1990).

Isotope studies in rats showed that 70% of an oral dose is expired as carbon dioxide within 12 hours (Siu & Draper 1982). The acute oral LD₅₀ of MDA in rats is 632µg/g body weight (Draper & Hadley 1990).

It is questionable whether these products would reach concentrations which might cause a clinical problem. TPN infused into a central vein is diluted by the rapid blood flow. For example, a 3kg baby receiving 3g/kg/day of lipid as 20% Intralipid has the infusion running at a rate 2.25ml per hour or 0.0375ml per minute. Each minute therefore the 0.0375ml of infusion is diluted in 255ml of blood, nearly a seven thousand-fold dilution. Whether this rate of infusion would cause any measurable effect has not yet been the subject of any clinical study. Given the low initial concentrations of lipid peroxides and MDA in the Intralipid, their subsequent dilution in the blood stream and mechanisms of

elimination, it seems unlikely that toxic levels would be reached. However no study has yet ruled out the possibility of toxicity in human neonates.

Different studies of lipid peroxide levels in parenteral nutrition solutions have used different assay methods. This makes it difficult to compare the results of the studies with each other or with my own results.

Wispe et al (1985) and Van Gossum et al (1988) report the presence of increased TBARS in lipid emulsions, but do not offer any figures. Marshall and Roberts (1990) found $5870 \pm 198 \mu\text{mol/l}$ of TBARS in 20% Intralipid. This figure is nearly 700 times the level of MDA found in my own studies. My own figures may be lower because of the greater specificity of the 1-methyl-2-phenylindole assay for MDA (Diaz 1998) than assays measuring TBARS.

Pitkanen et al (1991) reported $0.010 \mu\text{mol/l}$ MDA in 10% Intralipid. My own figures are 800 times greater than this. The method used to measure MDA is not given in the publication, but I would speculate that a different choice of assay has produced this large difference in results.

Marshall and Roberts(1990) reported that lipid peroxides were undetectable by an iodide titration assay. Helbock et al (1993) measured lipid hydroperoxide in 15 samples of 20% Intralipid by HPLC with chemiluminescence detection. The mean concentration was $290 \pm 29 \mu\text{mol/l}$ (SEM) lipid hydroperoxides, a figure 15 times my own. When measurements were made on Intralipid samples collected from the end of the intravenous tubing after a 20-hour infusion cycle the results were not significantly different from measurements made on newly opened bottles.

In contrast, Neuzil et al (1995), using the same assay methodology as Helbock et al, found that levels of triglyceride hydroperoxides in 20% Intralipid rose from a baseline $\sim 5.4\text{--}9.9\mu\text{M}$, to $27\text{--}77\mu\text{mol/l}$ after 24 hours ambient or phototherapy light exposure.

In order to decrease peroxidation in lipid emulsions Lavoie and Chessex (1997) studied the effect of iron additives on peroxidation in TPN (FeCl_2 , FeSO_4 , iron dextran & iron sorbitol) and recommended that bound iron should be used in PN for patients requiring supplementation.

Steger and Muhlebach (1997) compared the oxidation of i.v. lipid emulsions after storage in different admixture bags (made of ethyl-vinyl-acetate and poly-propylene:poly-amide) with oxidation after storage in bottles. In contrast to storage in glass bottles, significant peroxidation was detected in both emulsions after storage in the polymer bags. He therefore recommended that lipids in all-in-one bags should be stored light-protected in a refrigerator, with an oxygen-tight over-wrap when stored for extended periods.

The influence of vitamin E on the lipid peroxidation of i.v. lipid emulsions in TPN bags was investigated by the same group (Steger and Muhlebach 1998). Tocopherols are lipid soluble antioxidants *in vivo*. At concentrations of 20 mg/l, α tocopherol was an antioxidant as expected; however at a concentration 160 mg/l, the lipid peroxide levels were increased. This finding may have occurred because tocopherols can have a pro-oxidative effect in some circumstances. Tocopherols can reduce iron (III) to iron (II), which can act as a catalyst in reactions which generate free radicals. Also, during its action as an antioxidant, α tocopherol forms an α tocopherol radical. This radical may

either react with another free radical to form a non-radical product (thus performing an additional antioxidant action) or be recycled back to α tocopherol (Halliwell & Gutteridge 1999, p.215). However the α tocopherol radical may act as an initiator instead of a terminator of lipid peroxidation, which may account for the effect observed at the increased concentration.

A significant positive correlation was found between the concentrations of lipid peroxides and MDA in the emulsions. This was to be expected because MDA is a decomposition product of lipid peroxides.

6. Conclusions

Lipid peroxides and their breakdown products are present in intravenous lipid emulsions. Because of differing methodologies for their measurement, there is inconsistency in the levels observed by different authors. There is also disagreement over the effect of exposure to ambient light.

The correlation between lipid peroxide and MDA levels provides suggests that either of these indices alone provides a measure of lipid peroxidation in lipid emulsions.

3.2 IS FREE RADICAL ACTIVITY INCREASED IN INFANTS RECEIVING TPN?

1. Introduction

An increase in the indices of free radical activity in patients receiving TPN has been reported by several authors (Wispe et al 1985; Van Gossum et al 1988; Pitkanen et al 1991). These initial studies used expired breath pentane or ethane and pentane as indices of free radical activity. In the majority of my clinical studies only lipid peroxide and MDA production was measured. Although these products are frequently used as indices of free radical activity in clinical studies (e.g. Inder et al 1994), lipid peroxides and MDA had not been used in previous studies of the effect of TPN on free radical activity in infants.

2. Aim

The aim of this study was to compare free radical formation in patients receiving TPN with those not on TPN using lipid peroxides and MDA as indices of free radical activity.

3. Methods

Blood samples were obtained from 60 neonates and infants for plasma MDA and lipid peroxide measurement. The patients were less than 12 months of age and weighed less than 10kg. The patients were classified into those receiving enteral feeds, intravenous crystalloid solution or both and those receiving parenteral nutrition.

4. Results

29 infants were receiving enteral feeds/intravenous crystalloid and 31 were receiving parenteral nutrition. All were inpatients on the neonatal intensive care unit or surgical ward and were suffering from a diverse range of clinical problems. There was no significant difference in age between the two groups, but the group receiving TPN had a significantly lower gestational age and weight than those not requiring TPN (Table 3.2.1). There was no correlation between gestational age or weight and MDA in either patients who were or were not receiving TPN.

The mean plasma MDA concentration was $5.27\mu\text{mol/l}$ ($2.88\text{--}9.34\mu\text{mol/l}$) in the group receiving TPN and $2.85\mu\text{mol/l}$ ($1.01\text{--}6.26\mu\text{mol/l}$) in the group not receiving TPN. This difference was significant at the $p<0.0001$ level (Figure 3.2.1).

Lipid peroxides were measured in 20 of the patients not receiving TPN and 6 patients who were on TPN. The plasma lipid peroxide concentration was $4.39\mu\text{mol/L}$ ($1.88\text{--}10.55\mu\text{mol/L}$) in the group receiving TPN and $2.85\mu\text{mol/l}$ ($0.53\text{--}7.22\mu\text{mol/L}$) in the group not receiving TPN. There was no difference in lipid peroxide concentrations (Figure 3.2.2). There was no correlation between levels of MDA and lipid peroxides. ($r = 0$)

When the two groups were matched for gestational age and weight the MDA concentrations remained significantly different between the two groups ($p<0.005$).

	Enteral feeding/i.v. crystalloid n=29 mean (range)	TPN n=31 mean (range)
Gestation (weeks)	38 (28-42)	34 (23-40)*
Age (days)	54 (1-339)	67 (7-328)
Weight (kg)	3.9 (1.2-10)	2.8 (0.65-6.0)**

Table 3.2.1 Gestation, age and weight of patients in Study 2.

Significant difference, * $p < 0.0001$, ** $p < 0.001$

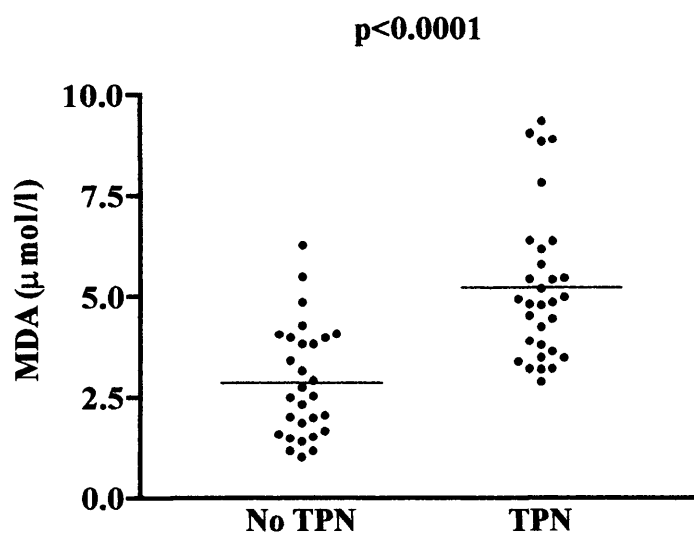


Figure 3.2.1 Plasma MDA concentrations in patients not receiving and receiving TPN.

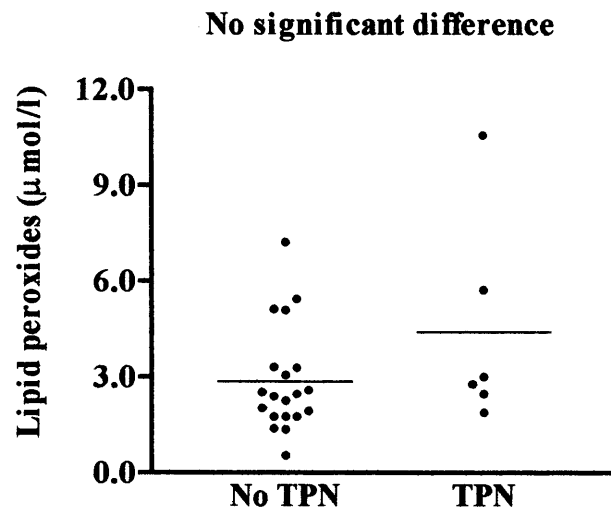


Figure 3.2.2 Plasma lipid peroxide levels in patients not receiving and receiving TPN.

5. Discussion

The results from this study showed that MDA concentrations are significantly higher in patients receiving TPN than those receiving enteral feeds and/or intravenous crystalloid infusions. This supports the hypothesis that patients receiving TPN are subject to increased free radical formation compared with those not receiving TPN, and is in agreement with the findings of other studies (Wispe et al 1985; Pitkanen et al 1991).

In this study the group receiving TPN had a lower gestational age ($p < 0.0001$) and weight ($p < 0.001$) than those not requiring TPN. It is conceivable that these parameters, or that a related confounding factor may influence MDA levels. However no correlation was found between gestational age and weight in this or in other studies (Yigit et al 1998). Possible confounding factors include the administration of O_2 and differing clinical states between the groups. No

infant in the study was receiving supplementary O₂. It is possible that clinical differences between the groups may have caused the observed difference. The effect of illness severity on free radical formation was therefore subject to a separate study (Study 6).

There was no significant difference in plasma lipid peroxide concentrations between the two groups and there was no correlation between MDA and lipid peroxide concentrations. Such a lack of correlation between plasma lipid peroxides and MDA has been observed by other workers (Nourooz-Zadeh et al 1994).

Lipid peroxides are the initial product of free radical attack upon a polyunsaturated fatty acid. Lipid peroxides undergo further reactions and degrade to form other products (including MDA). It is possible that *in vivo*, lipid peroxides undergo further reactions and degradation which does not occur in the lipid emulsions. Another reason for the lack of correlation may be because of the unpredictable loss of lipid peroxides which has been observed during the storage of plasma samples (Sodergren et al 1998).

6. Conclusions

Free radical activity is increased in neonates receiving parenteral nutrition compared with those not receiving parenteral nutrition.

Plasma lipid peroxide concentrations did not correlate with MDA concentrations in this *in vivo* study.

3.3 DOES THE CESSATION OF THE LIPID INFUSION DECREASE FREE RADICAL ACTIVITY?

1. Introduction

The generation of oxygen-derived free radicals during TPN has been ascribed to the lipid component of the TPN regimen (Wispe et al 1985; Van Gossum et al 1988; Pitkanen et al 1991). Previous studies have shown that stopping the lipid infusion resulted in a decrease in expired breath pentane and therefore, by implication free radical activity (Wispe et al 1985; Pitkanen et al 1991). No studies have been performed to measure the effect of stopping the lipid infusion using MDA as an index of free radical activity.

Indirect calorimetry was performed to determine the effect of stopping the lipid infusion on substrate utilization.

2. Aim

The aim of this study was to determine the effect of discontinuing the lipid infusion on lipid utilization and MDA concentrations in post-operative surgical infants.

3. Methods

Metabolic studies were performed over two days on 6 surgical infants receiving post-operative TPN (Table 3.3.1). Their mean age was 51 days and the mean time receiving TPN was 14 days. The study design is shown in figure 3.3.1. On the first day a standard TPN regimen was administered which provided 18 g/kg/day of carbohydrate and 3g/kg/day of lipid. On the second day

the carbohydrate infusion remained at 18g/kg/day and the lipid infusion was discontinued. The volume infused and amounts of all other constituents in the TPN were kept constant over the period of the study. On each day respiratory gas exchange was measured by indirect calorimetry. Calorimetry was performed for two hours at the same time each day. Calorimetry was not available for Patient 5 because of malfunction of the calorimeter. A blood sample was obtained at the same time each day for estimation of plasma MDA concentrations. The FOX assay for measurement of lipid peroxides was still being developed at the time of this study.

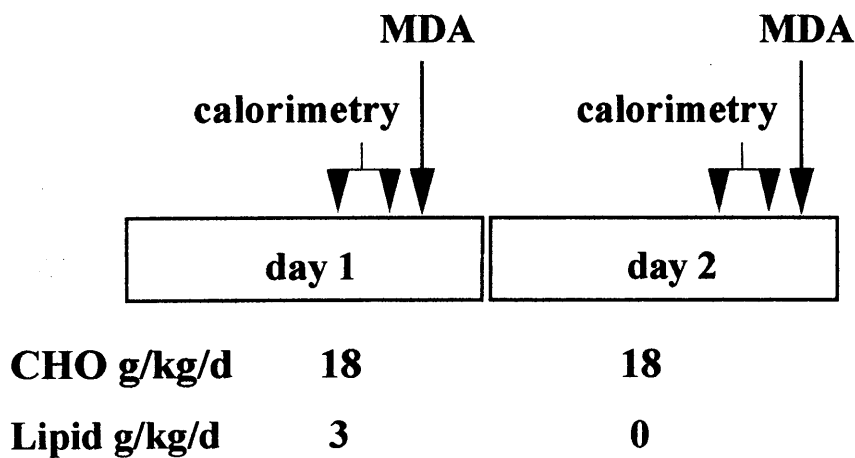


Figure 3.3.1 Experimental design for Study 3. On Day 1 a standard TPN regimen was administered. On Day 2 the lipid was discontinued. Indirect calorimetry was performed for two hours at the same time each day. A blood sample was obtained for measurement of plasma MDA at the same time each day.

4. Results

There was no significant change in $\dot{V}O_2$, $\dot{V}CO_2$ (Table 3.3.2) net lipid utilization and resting energy expenditure (Table 3.3.3) over the two consecutive days of the study (paired t-test).

The plasma MDA concentration decreased from a mean of $6.70\mu\text{mol/l}$ on the first day to $6.13\mu\text{mol/l}$ on the second day, when no lipid was administered (Table 3.3.4, Figure 3.3.2). When the paired data on each patient was compared the decrease in MDA concentrations was significant at the $p<0.01$ level (mean difference 0.57; 95% CI 0.24-0.91).

Patient	Gestation (weeks)	Weight (kg)	Age (days)	Time on TPN (days)	Diagnosis
1	34	2.87	66	4	Gastroschisis
2	32	1.80	17	11	Malrotation
3	32	2.50	57	38	Meconium peritonitis
4	33	1.79	10	8	Necrotizing enterocolitis
5	35	3.60	110	24	Malrotation
6	31	1.60	47	3	Necrotizing enterocolitis

Table 3.3.1 Gestation, age, weight and diagnosis of patients in Study 3.

Patient	$\dot{V}O_2$ ml/kg/min		$\dot{V}CO_2$ ml/kg/min	
	Day 1	Day 2	Day 1	Day 2
1	7.25	9.13	7.03	9.23
2	7.28	6.72	7.72	7.50
3	7.04	7.28	7.12	7.36
4	7.54	7.43	7.71	8.16
5	N/A	N/A	N/A	N/A
6	4.19	6.06	4.19	6.25

Table 3.3.2 Oxygen consumption and carbon dioxide production of patients in Study 3.

N/A = Not available due to calorimeter malfunction

Patient	Lipid utilization g/kg/day		EE kcal/kg/day	
	Day 1	Day 2	Day 1	Day 2
1	0.39	-0.38	52.75	66.97
2	-1.21	-2.03	54.11	50.63
3	-0.33	-0.33	51.74	53.49
4	-0.55	-1.91	55.53	55.70
5	N/A	N/A	N/A	N/A
6	-0.14	-0.60	30.89	44.81

Table 3.3.3 Lipid utilization and energy expenditure of patients in Study 3.

Negative values for lipid utilization represent fat synthesis.

N/A = not available

Patient	MDA $\mu\text{mol/l}$	
	Day 1	Day 2
1	6.89	5.78
2	4.85	4.24
3	8.89	8.68
4	4.23	3.56
5	9.34	9.04
6	6.00	5.47

Table 3.3.4 Plasma MDA of patients in Study 3.

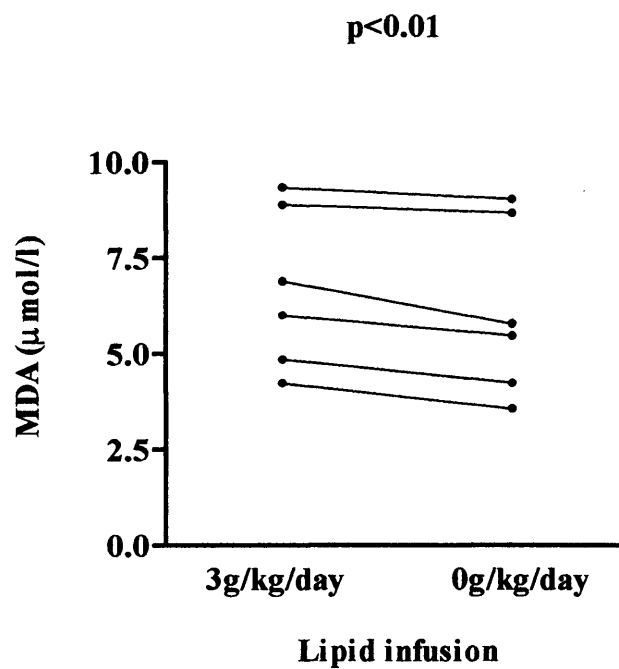


Figure 3.3.2 The change in plasma MDA on cessation of the lipid infusion.

5. Discussion

This study indicated that cessation of the lipid infusion resulted in a decrease in free radical activity.

The administration of Intralipid may result in free radical activity in the following ways: i) by providing the substrates (polyunsaturated fatty acids), and/or ii) by providing the initiators of free radical reactions (carbon centred radicals derived from lipid peroxides).

As described previously, the presence of some of the indices of free radical activity has been reported in the TPN solutions themselves and early reports considered the possibility that increased levels of the products of lipid peroxidation *in vivo* simply reflected their administration in TPN (Wispe et al 1985; Van Gossum et al 1988). Pitkanen et al (1991), using expired pentane as an index of free radical activity, concluded, however that lipid peroxidation was occurring *in vivo*. My studies are in agreement with this conclusion. In Study 1 the mean concentration of MDA in Intralipid was $8.69\mu\text{mol/l}$; if the infused lipid were the only source of MDA, then the dilutional effect of the plasma would be expected to result in a plasma concentration several times lower than that observed in the infusate. However the mean plasma MDA concentration observed during lipid infusion in this study was $6.70\mu\text{mol/l}$. These studies were performed over only two days. It is possible that by prolonging the lipid free period a further change in MDA concentrations may be achieved over time.

Indirect calorimetry on this group of patients showed no difference in the energy expenditure over the two days, suggesting that the infants were clinically stable. There was also no significant difference in lipid utilization despite the

fact that no lipid emulsion was infused on the second day. This may indicate that fat utilization rates are independent of the source of fat, which may be exogenous (from food or TPN) or endogenous (from fat stores). Studies have shown that lipid utilization is favoured by the post-traumatic or post-surgical hormonal response. In spite of high glucose levels free fatty acid turnover is increased in post-trauma patients. Endogenous lipids are mobilised by hormone sensitive lipase activated by raised plasma glucagon and adrenaline (Nordenstrom et al 1983).

6. Conclusions

The studies have confirmed that a decrease in free radical activity in infants receiving parenteral nutrition may be achieved by cessation of the lipid infusion.

3.4 DOES PROMOTING LIPID UTILIZATION INFLUENCE FREE RADICAL ACTIVITY IN INFANTS ON TPN?

1. Introduction

Previous studies have demonstrated that increased free radical activity is observed in infants receiving TPN and that this increase is linked to the lipid infusion.

It has been hypothesised that if the exogenous lipid emulsion is completely oxidised, i.e. expired as carbon dioxide and excreted as water there should be a reduced risk of undesirable side effects (Schmidt-Sommerfeld et al 1982). Pierro et al (1989) have demonstrated that surgical neonates are able to oxidize up to 83% of the lipid infused intravenously. Metabolic studies on surgical newborn infants receiving TPN including carbohydrate, amino acids and lipid have shown that net lipid oxidation is influenced by carbohydrate intake and energy expenditure and minimally affected by the lipid intake (Pierro et al 1993). A close negative correlation between intravenous fat oxidation and carbohydrate intake has been observed in post-trauma patients and in post operative surgical neonates (Letton et al 1996).

2. Aim

To test the hypothesis that a reduction of the carbohydrate/lipid ratio in the TPN regimen will result in increased utilization of lipid and consequently a decreased production of free radicals.

3. *Methods*

Metabolic studies were performed on 17 surgical infants whose details are provided in Table 3.4.1. All the infants were free from sepsis, received no enteral feeds and were clinically stable over the course of the study. The patients were receiving TPN because of postoperative gut dysfunction or in the recovery phase from necrotizing enterocolitis. The study started at least 3 days after the operation or after the diagnosis of necrotizing enterocolitis, when the patient's general condition had stabilised. During the study each patient received continuous TPN. The intravenous fluid intake varied from 100 to 180 ml/kg/day according to the patient's requirement.

The experiments were performed over two consecutive days (Figure 3.4.1).

Over the two study days, the lipid infusion was kept constant at 3g/kg/day and the carbohydrate infusion was changed from 18g/kg/day on day one to 10g/kg/day on day two in order to increase the lipid utilization.

On each day respiratory gas exchange was measured by indirect calorimetry (15 patients) and a blood sample obtained for estimation of plasma MDA concentrations.

4. *Results*

There was no significant change in $\dot{V}O_2$, $\dot{V}CO_2$ (Table 3.4.2) and resting energy expenditure (Table 3.4.3) between the two consecutive days of the study.

The net lipid utilization (Table 3.4.3, Figure 3.4.2) increased significantly on the second (low carbohydrate) day. When the data on each individual patient

was compared by a paired t-test the difference was significant at the $p < 0.0001$ level (mean difference 1.65; 95% CI 0.94-2.37).

The MDA concentration (Table 3.4.4, Figure 3.4.3) decreased on the second day. When the paired data on each patient was compared the decrease in MDA concentrations was significant at the $p < 0.01$ level (mean difference 0.75; 95% CI 0.21-1.28).

There was no significant correlation between the change in lipid utilization and the change in MDA (Figure 3.4.4).

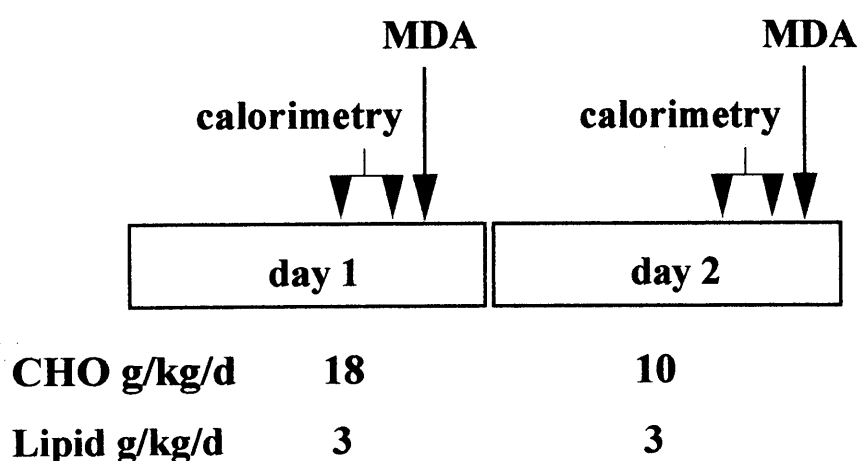


Figure 3.4.1 Experimental design for Study 4. On Day 1 a standard TPN regimen was administered. On Day 2 the carbohydrate administered was reduced from 18g/kg/day to 10g/kg/day. Indirect calorimetry was performed for two hours at the same time each day. A blood sample was obtained for measurement of plasma MDA at the same time each day.

Patient	Gestation (weeks)	Weight (kg)	Age (days)	Time on TPN (days)	Diagnosis
1	38	2.35	9	7	Necrotizing enterocolitis
2	30	3.72	134	132	Necrotizing enterocolitis
3	33	3.50	328	189	Persistent diarrhoea
4	37	3.30	11	10	Gastroschisis
5	38	3.70	63	30	Gastroschisis
6	38	4.20	75	42	Gastroschisis
7	37	2.50	23	20	OA, TOF, duodenal atresia
8	36	4.80	212	50	Gastroschisis, ileal atresia
9	40	5.00	231	134	Microvillous atrophy
10	40	3.20	23	11	Malrotation
11	39	3.00	21	8	Necrotizing enterocolitis
12	34	2.87	65	3	Gastroschisis
13	32	1.70	11	5	Malrotation
14	31	2.07	47	8	Duodenal atresia
15	37	2.03	14	12	OA & TOF
16	35	3.60	110	22	Malrotation
17	36	3.72	91	11	Poor growth

Table 3.4.1 Gestation, age, weight and diagnosis of patients in Study 4.

OA = oesophageal atresia, TOF = tracheo oesophageal fistula

Patient	$\dot{V}O_2$ ml/kg/min		$\dot{V}CO_2$ ml/kg/min	
	Day 1	Day 2	Day 1	Day 2
1	8.58	9.17	8.74	8.40
2	6.64	10.82	6.59	9.45
3	15.26	17.17	14.29	14.20
4	7.60	6.73	7.00	6.73
5	8.78	8.12	8.27	7.49
6	8.33	7.62	8.29	7.55
7	3.43	3.29	3.42	3.14
8	11.02	10.20	9.94	8.60
9	10.40	10.82	10.20	9.52
10	9.44	7.41	9.06	6.99
11	6.68	7.46	6.79	7.02
12	7.25	8.68	7.03	7.77
13	5.05	6.12	5.80	6.24
14	7.77	7.10	8.26	6.67
15	N/A	N/A	N/A	N/A
16	N/A	N/A	N/A	N/A
17	5.13	3.99	5.13	3.54

Table 3.4.2 Oxygen consumption and carbon dioxide production of patients in Study 4.

Patient	Lipid utilization g/kg/day		EE kcal/kg/day	
	Day 1	Day 2	Day 1	Day 2
1	-0.51	1.72	63.13	65.75
2	-0.02	3.18	48.61	76.71
3	2.21	7.06	109.67	120.09
4	1.31	1.78	54.64	47.99
5	1.09	1.39	63.37	58.36
6	-0.04	0.03	60.90	55.70
7	-0.12	0.22	25.35	24.09
8	2.48	3.74	78.66	71.81
9	0.34	3.01	75.68	76.83
10	0.78	0.88	68.39	53.57
11	-0.41	0.92	49.18	53.90
12	0.39	2.06	52.75	61.95
13	-1.96	-0.43	38.44	45.12
14	-1.33	0.90	57.76	51.30
15	N/A	N/A	N/A	N/A
16	N/A	N/A	N/A	N/A
17	-0.14	0.95	37.72	28.66

Table 3.4.3 Lipid utilization and energy expenditure of patients in Study 4.

Negative values for lipid utilization represent fat synthesis. N/A = not available

Patient	MDA $\mu\text{mol/l}$	
	Day 1	Day 2
1	6.37	3.80
2	4.80	5.70
3	3.38	2.21
4	2.14	2.88
5	3.63	2.96
6	3.61	3.37
7	3.47	2.22
8	6.97	4.78
9	3.88	3.98
10	5.45	3.64
11	3.48	3.81
12	6.99	5.41
13	6.38	4.95
14	4.43	4.53
15	7.82	6.23
16	9.34	9.40
17	4.52	4.12

Table 3.4.4 Plasma MDA of patients in Study 4.

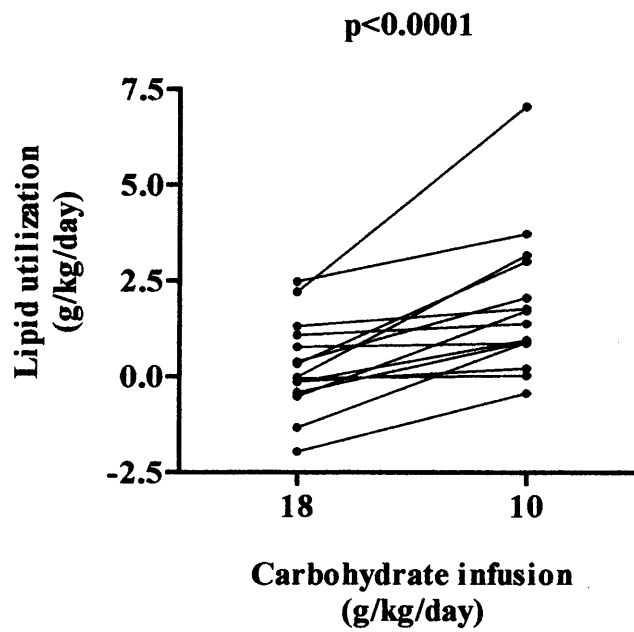


Figure 3.4.2 The change in lipid utilization when the carbohydrate administration was reduced. The net lipid utilization increased significantly when the infused carbohydrate was decreased.

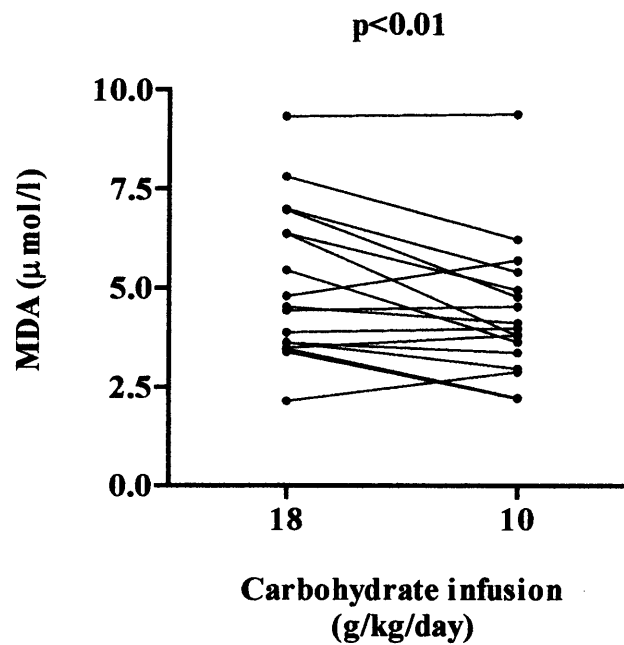


Figure 3.4.3 The change in MDA which occurred when the carbohydrate administered was reduced. The plasma MDA concentration decreased significantly when the infused carbohydrate was decreased.

No Correlation

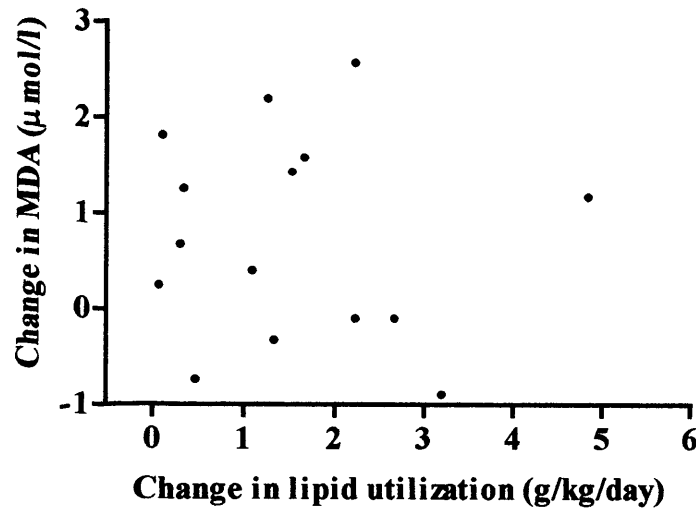


Figure 3.4.4 Correlation between the changes in lipid utilization and MDA concentrations

5. Discussion

The results of Study 3 showed that stopping the lipid infusion resulted in a fall in free radical activity in infants receiving TPN. Because of some of the complications associated with intravenous lipids it has been suggested that they should not be used in vulnerable groups (Cooke 1991). However the administration of intravenous lipid offers several advantages. As a solution of low osmolarity, it provides a rich source of calories in a relatively small volume and it is the only source of essential fatty acids for patients on TPN. The human neonate makes a transition at birth from deriving most of their energy from glucose to deriving a large proportion from fat. There is a rise in circulating free fatty acids and a fall in the respiratory quotient (Bourgnères 1982, Sauer 1994).

In breast milk and formula feeds up to 50% of total calories are provided as fat (Van Aerde 1998). Studies in adults have shown increased lipid oxidation following surgery and trauma (Carpentier 1981), though this increase may not occur in neonates (Jones 1993). It would therefore be desirable if the lipid derived free radical activity could be reduced without reducing the lipid infusion itself. In this study I tested the hypothesis that if lipid utilization was increased, a decrease in free radical activity would be obtained.

It has been shown that when carbohydrate intake increases, the proportion of fat oxidized decreases (Thiebaud 1982, Letton 1996) and that glucose intake is the principal determinant of glucose and lipid utilization in neonates receiving TPN (Jones 1993). Therefore lipid utilization was increased by decreasing the carbohydrate administered in this study.

Lipid utilization may be considered in two stages - clearance of the lipid emulsion particles from the circulation and intracellular fatty acid oxidation. Decreased carbohydrate availability acts at both of these stages. Intralipid is carried in the circulation as particles of similar size to chylomicrons which are cleared by lipoprotein lipase in the same way (Van Aerde et al 1998). The activity of this enzyme in some tissues increases during fasting (Hamosh & Hamosh 1983), therefore its activity may increase in response to the decreased carbohydrate provision. Fatty acid oxidation increases due to a decrease in plasma insulin and a decrease in the availability of glucose.

The results of this study showed that a reduction in the carbohydrate/lipid ratio in the TPN regimen resulted in a significant increase in lipid utilization and a significant decrease in free radical mediated MDA

formation. Therefore it may not be necessary to discontinue the infusion of the lipid to obtain a reduction in TPN associated free radical activity.

There was no significant correlation between the increase in lipid utilization and the reduction in MDA. Possible reasons for this include: i) there is no dose-response relationship between the two variables, ii) there were insufficient numbers of patients or the duration of the experiment was too short to reveal a relationship or iii) the changes in the lipid utilization and MDA are not directly linked and occurs for some other reason.

Intravenous lipid administration may result in increased free radical activity by providing: i) the substrates (polyunsaturated fatty acids) or ii) the initiators of free radical reactions (carbon centred radicals derived from fatty acids). The results of this experiment suggest that as lipid utilization is increased a greater proportion of initiators and substrates will be removed from the circulation, resulting in a decrease in free radical activity.

Our studies were performed over only two days. It is possible that by prolonging the low carbohydrate diet a further decrease in MDA concentrations may be achieved over time. Further studies are needed to explore this possibility.

6. Comparison of the results of lipid exclusion and promotion of lipid utilization

The decrease in plasma MDA resulting from discontinuing the lipid infusion (mean difference 0.57 ± 0.13) was similar to that obtained by promoting lipid oxidation (mean difference 0.75 ± 0.25). There was no significant difference when these results were compared.

7. Conclusions

It is not necessary to stop the infusion of lipid in order to reduce the production of oxygen derived free radicals. Promoting lipid utilization by reducing the carbohydrate/lipid ratio of TPN reduces the production of oxygen derived free radicals to a similar extent to lipid exclusion. Manipulation of the carbohydrate/fat ratio may therefore be a powerful tool in changing the metabolism of fat infusions to reduce their toxic effects while allowing continued administration.

3.5 IS TPN ASSOCIATED FREE RADICAL ACTIVITY INFLUENCED BY USING A MEDIUM CHAIN TRIGLYCERIDE LIPID INFUSION?

1. Introduction

The most commonly used intravenous lipid emulsions contain predominantly long chain triglycerides (LCT) with fatty acid carbon chain lengths of 16 to 20 atoms. However studies have suggested advantages of infusing a lipid emulsion based on a mixture of LCT and medium chain triglycerides (MCT), the latter containing fatty acids of 6 to 12 carbon atoms chain length.

MCTs are cleared from the bloodstream at a faster rate (Wolfram 1986) and are oxidised more completely than LCT (Johnson et al 1990). It has been postulated that the reason for these findings is that lipoprotein lipase has a greater affinity for MCT and that once in the cell, medium chain fatty acids do not require a carrier system to enter the mitochondrion (Metges & Wolfram 1991).

On the basis of the results obtained in Study 4 I hypothesised that the administration of an MCT containing formula may be associated with a decrease in free radical formation because of its faster clearance and oxidation.

2. Aim

The aim of this study was to compare the free radical activity in neonates when receiving a 100% LCT emulsion and during infusion of an emulsion containing a mixture of 50% LCT and 50% MCT.

3. Methods

The studies were conducted over two consecutive days on 7 neonates receiving total parenteral nutrition (TPN) for gut dysfunction following surgery for meconium ileus, necrotizing enterocolitis, duodenal atresia or diaphragmatic hernia. The patient's details are in table 3.5.1. The patients had been receiving TPN for 3 to 6 days. Intravenous fluid intake varied from 100 to 180 ml/kg/day according to the patient's requirements.

All the patients were receiving 20% Intralipid as part of their TPN regimen. For a single day a 20% 50/50 MCT/LCT emulsion (B.Braun, Melsungen, Germany) was administered and then the original 20% Intralipid infusion was resumed. The volume of the infusion and the quantity of carbohydrate, amino acids and fat (g/kg/day) were kept constant over the period of the study. On each day blood samples were obtained for plasma MDA and lipid peroxide estimation. In three patients breath samples were obtained for pentane analysis (Figure 3.5.1).

4. Results

The administration of a pure LCT emulsion was associated with significantly lower levels of MDA than when an MCT/LCT emulsion was used (Figure 3.5.2; $p < 0.05$). The pentane excretion was also reduced on the LCT day in the three patients in whom it was measured (Figure 3.5.3) Because values were obtained in only 3 patients statistical analysis was not performed. There was no significant difference in the lipid peroxide levels between the two days (Figure 3.5.4).

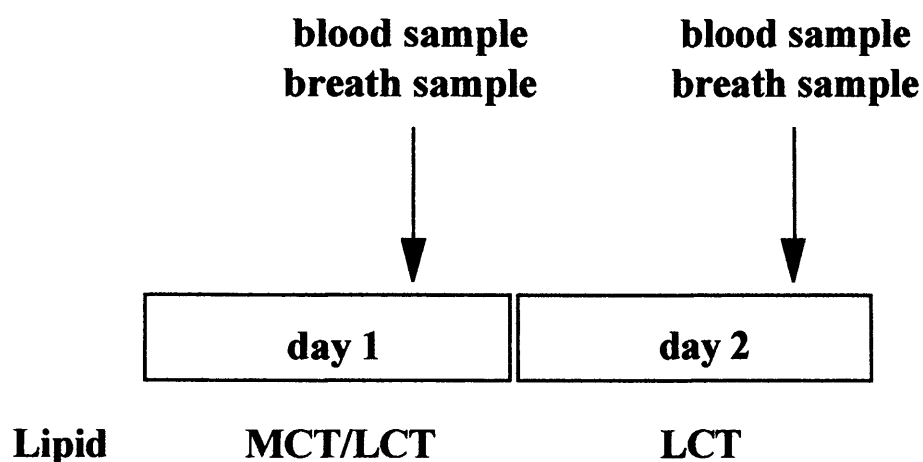


Figure 3.5.1 Experimental design for Study 5. On Day 1 the lipid component of the TPN regimen consisted of a 50/50 MCT/LCT emulsion. On Day 2 the lipid infusion consisted of LCT only. A blood sample was obtained for measurement of plasma MDA and lipid peroxides at the same time each day. In 3 patients breath samples were obtained for the measurement of expired breath pentane.

Patient	Gest (weeks)	Weight (kg)	Age (days)	Time on TPN (days)	Diagnosis
1	30	3.07	122	3	Cystic fibrosis
2	35	2.35	5	4	Meconium peritonitis
3	27	1.40	45	3	Necrotizing enterocolitis
4	42	4.20	11	5	Diaphragmatic hernia
5	31	1.37	5	3	Duodenal atresia
6	32	2.83	20	3	Necrotizing enterocolitis
7	31	2.00	11	6	Necrotizing enterocolitis

Table 3.5.1 Gestation, weight, age and diagnosis of patients in Study 5

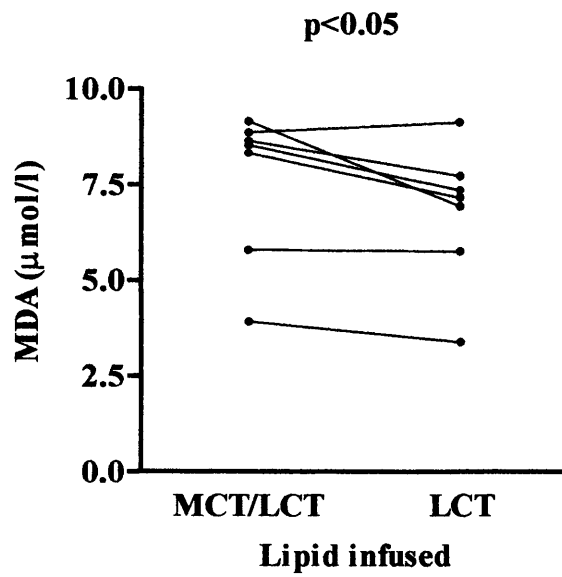


Figure 3.5.2 The change in plasma MDA concentrations on changing the lipid infusion from a medium and long chain triglyceride to a pure long chain triglyceride emulsion.

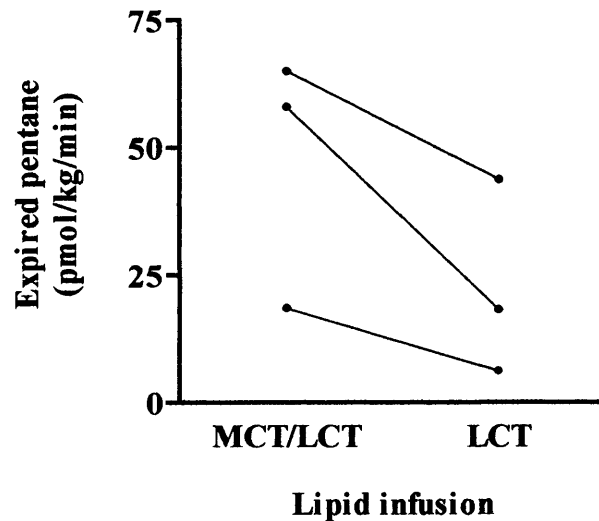


Figure 3.5.3 The change in expired breath pentane concentrations on changing the lipid infusion from a medium and long chain triglyceride to a pure long chain triglyceride emulsion.

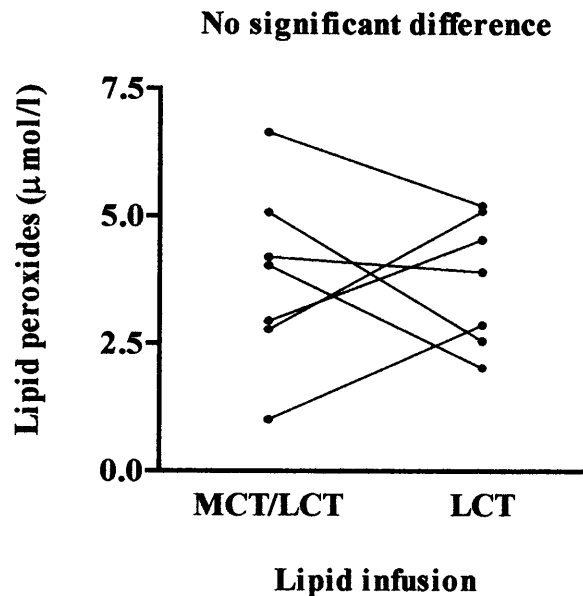


Figure 3.5.4 The change in plasma lipid peroxide concentrations on changing the lipid infusion from a medium and long chain triglyceride to a pure long chain triglyceride emulsion.

5. Discussion

The results of this study show that plasma MDA concentrations in patients receiving TPN is increased if a 50/50 MCT/LCT lipid emulsion is used instead of a formula containing only LCT.

In Study 4 the results showed that plasma MDA (and by implication, free radical formation) during TPN was reduced by promoting lipid oxidation. MCTs are cleared from the bloodstream at a faster rate (Wolfram 1986) and are oxidised more completely than LCT (Johnson et al 1990). My hypothesis was that the MCT/LCT emulsion would result in a reduction in free radical activity compared with an LCT emulsion. However free radical formation was increased during the MCT/LCT infusion.

This effect may be the result of increased neutrophil free radical production. Oxygen derived free radicals are produced during bacterial killing by phagocytic white blood cells. These cells show a large increase in oxygen metabolism when activated which has been termed the “respiratory burst” (Lunec 1990). Superoxide, hydrogen peroxide and the hydroxyl radical are generated by electron transfer from NADPH to oxygen. The reactive oxygen species are toxic to ingested bacteria. One study of the effect of lipid emulsions on neutrophil function concluded that the early respiratory burst is accelerated by the MCT/LCT emulsion but unaffected by a pure LCT emulsion (Wanten et al 1999). It is possible that medium chain triglycerides interact directly with neutrophils to stimulate free radical production *in vivo* and that this has resulted in the effect we have observed. However a similar study (Wu et al 1999) found that all types of lipid emulsion (LCT, MCT/LCT and structured triglyceride) caused a reduction in neutrophil superoxide production and that there was no difference between the different emulsions in this effect.

Another possible explanation for the result of this experiment is suggested by the results of Studies 2 and 3. The increased lipid utilization which accompanies MCT/LCT administration may be due mainly to oxidation of the MCT component of the solution. This may leave the LCT component relatively under utilized and therefore available to contribute to further free radical activity.

6. Conclusions

The administration of a 50:50 MCT:LCT lipid infusion results in an increase in the indices of free radical activity. This may occur due to an increase

in neutrophil free radical production or increased free radicals derived from the LCT component of the infusion.

3.6 IS THERE A RELATIONSHIP BETWEEN FREE RADICAL ACTIVITY AND ILLNESS SEVERITY?

1. Introduction

An increase in the formation of oxygen derived free radicals has been observed in inflammatory disorders (such as ulcerative colitis (Babbs 1992) and rheumatoid arthritis (Edmonds et al (1997)), infections and sepsis (Novelli 1997). This may be caused by free radical production which accompanies the events associated with inflammation, such as the “respiratory burst” of white cells during bacterial killing, ischaemia/reperfusion episodes and during the metabolism of arachidonic acid to prostaglandins and leukotrienes (Lunec 1990).

Free radical activity has been correlated with an adverse outcome in very low birthweight infants (Inder et al 1996; Nycyk et al 1998). Severity of illness has been quantified with plasma markers such as cytokines (van Deuren et al 1995) and physiological scoring systems such as the PRISM score (Pollack et al 1988). As free radicals are known to be mediators in the pathogenesis of various disorders, their level of activity may also serve as a marker for the severity of the illness. However there are no studies examining whether free radical activity is related to any measure of illness severity.

2. Aim

To establish whether there is a relationship between free radical activity and severity of illness.

3. *Methods*

Studies were performed on 60 neonates and infants (Table 4.6.1 and 4.6.2) aged less than 12 months of age and weighing less than 10kg. Three categories of patients were studied: a) controls: these patients were undergoing elective surgery for cleft lip or inguinal hernia repair (Group 1, n=8); b) patients with major gastrointestinal abnormalities recovering from surgery on a general paediatric surgery ward (n=24). 10 of these patients were receiving enteral feeds or i.v. fluids (Group 2) and 14 were receiving TPN (Group 3); c) patients in the Neonatal Intensive Care Unit (NICU) who were recovering from major thoracic or abdominal surgery or had respiratory disorders (n=28). 11 of these patients were receiving enteral feeds or intravenous crystalloid fluids (Group 4) and 17 were receiving TPN (Group 5). Post-operative patients were studied a median of 7 (range 1-141) days after surgery. Blood samples were taken to measure the plasma free radical activity and cytokines. Control patients were studied on the day of admission to the hospital. Blood white cell counts (WCC) and C-reactive protein (CRP) levels were recorded where they were available. The Paediatric Risk of Mortality (PRISM) score was calculated for patients in NICU.

4. *Results*

The control patients were older and had a greater gestational age and weight than either the ward or the NICU patients, $p<0.05$, (Table 3.6.1). MDA concentrations were significantly lower ($p<0.001$) in controls compared to both ward and NICU patients (Figure 3.6.1). MDA concentrations were also significantly lower ($p<0.001$) in ward patients compared to NICU patients. Patients receiving TPN in the ward or in the NICU had significantly greater

plasma MDA concentrations ($p<0.001$) than patients receiving intravenous crystalloid and/or enteral feeding (Figure 3.6.2a and b). There was no significant difference in the WCC between the no-TPN (Group 2) and TPN (Group 3) ward patients. Neither was there any significant difference in any of the illness severity measures (WCC, TNF- α and IL-6, CRP, PRISM score) between the no-TPN (Group 4) and TPN (Group 5) NICU patients (Tables 3.6.2 and 3.6.3).

There was no significant difference in TNF- α among the study groups (Table 3.6.2). IL-6 was lower in the control group compared to patients in NICU ($p<0.01$). There was no significant relationship between MDA and TNF- α or between MDA and IL-6 when the results of all the patients were analysed. However when those patients receiving TPN are excluded from the analysis there was a significant correlation between concentrations of MDA and TNF- α ($r=0.54$, $p<0.05$) and between MDA and IL-6 concentrations ($r=0.74$, $p<0.001$; Figure 3.6.3a and b).

The mean PRISM score on admission to NICU ($n=27$) was 13 (4-38) and on the day of blood sampling ($n=18$) was 12 (3-22). There was no relationship between plasma MDA concentration and the PRISM score at admission or at the time of the study.

	Group 1 (n=8) Controls	Group 2 (n=10) Surgery no TPN	Group 3 (n=14) Surgery + TPN	Group 4 (n=11) NICU no TPN	Group 5 (n=17) NICU + TPN
Gestation (weeks)	40 (37-42)	39 (28-42)	36 (30-40)	39 (35-41)	32 (23-39)
Weight (kg)	5.3 (2.9- 10.0)*	3.0 (1.2-5.2)	3.4 (1.6-6.0)	3.7 (2.4-4.4)	2.1 (0.7-3.6)
Age (days)	91 (40- 260)*	21 (2-339)	64 (11-328)	7(1-50)	21 (7-112)

Table 3.6.1 Gestation, weight and age of patients in Study 6.

Data are expressed as mean (range).

*Significantly greater than other groups ($p < 0.05$)

	Group 1 (n=8) Controls	Group 2 (n=10) Surgery no TPN	Group 3 (n=14) Surgery + TPN	Group 4 (n=11) NICU no TPN	Group 5 (n=17) NICU + TPN
WCC (x 10⁹/l)	N/A	9.7 (6.5- 15.0)	8.2 (5.3- 17.4)	9.5 (6.8- 19.3)	10.5 (4.3- 18.6)
TNF-α (pg/ml)	7.4 (0-242)	42.13 (0- 142)	N/A	15.5 (1.6- 25.6)	32.4 (3.7- 68.1)
IL-6 (pg/ml)	2.0 (0-113)	45.67 (4.0- 68.2)	N/A	66.7 (119- 289)	32.8 (0.7- 150)
MDA (μmol/l)	1.5 (1.0-4.0)	2.7 (1.4-4.3)	4.2 (3.2-6.0)	4.0 (2.0-6.3)	5.8 (2.9-9.3)
PRISM Score	N/A	N/A	N/A	13 (4-26)	13 (4-38)
CRP (mg/l)	N/A	N/A	1.1 (0.0-9.2)	1.9 (0-8.3)	2.5 (0-6.3)

Table 3.6.2 White cell count, TNF-α, IL-6, MDA, PRISM score and CRP of patients in Study 6. Data are expressed as mean (range).

N/A = not available.

TNF-α and IL-6 were not measured for Group 3 because of insufficient blood

WCC and CRP were only available when measured for clinical reasons,

therefore were unavailable for Group 1 patients.

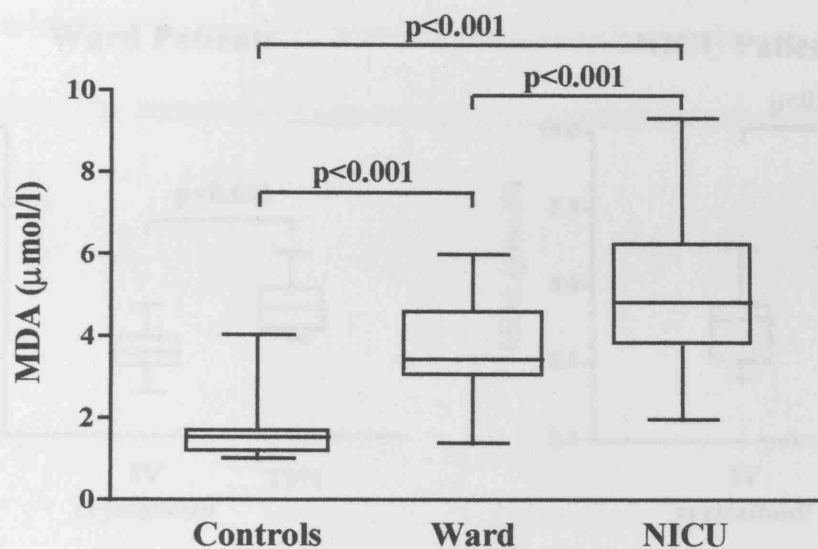


Figure 3.6.1 Plasma MDA concentrations in control, ward and neonatal intensive care patients.

The box extends from the 25th percentile to the 75th percentile, the horizontal line is at the median, the whiskers show the range.

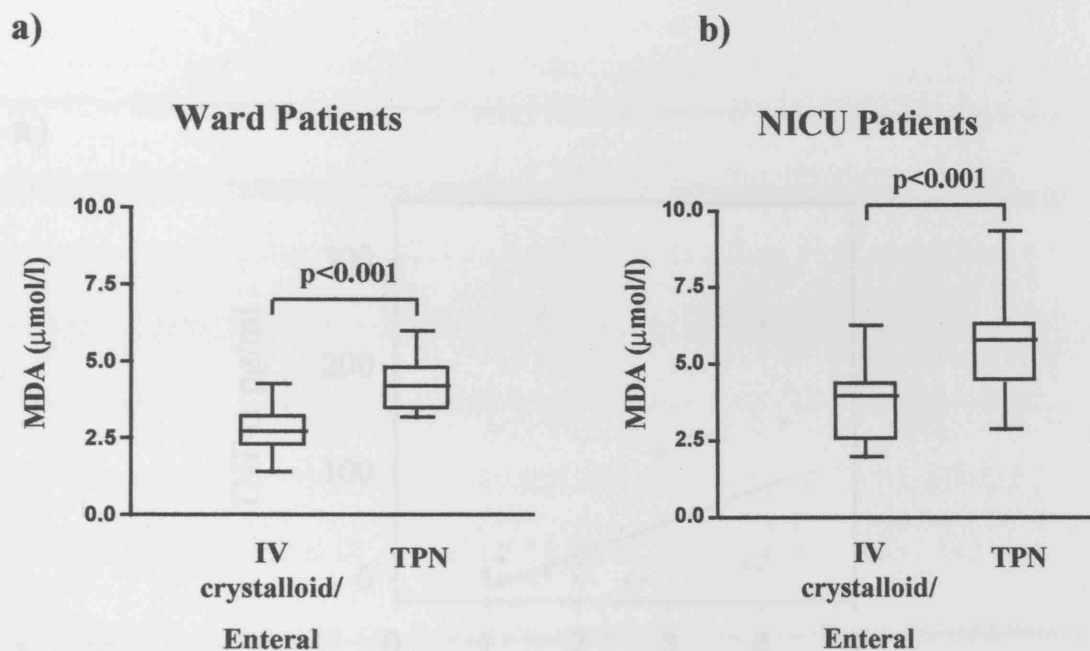
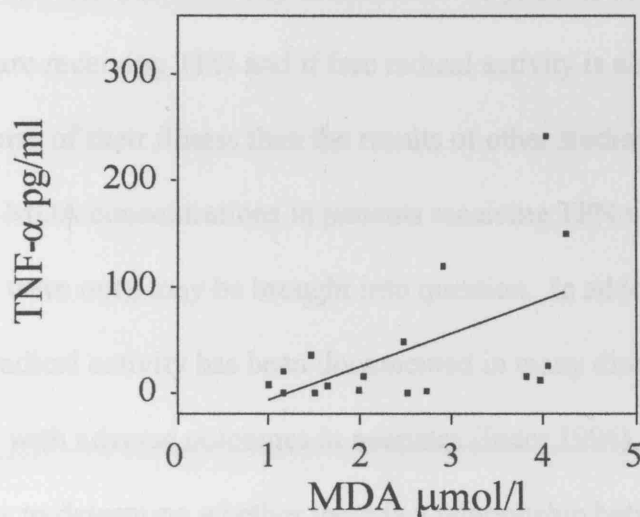


Figure 3.6.2 The comparison of MDA concentrations of a) ward and b) NICU patients receiving either TPN or enteral feeding and/or intravenous crystalloid solutions.

The box extends from the 25th percentile to the 75th percentile, the horizontal line is at the median, the whiskers show the range.

a)



b)

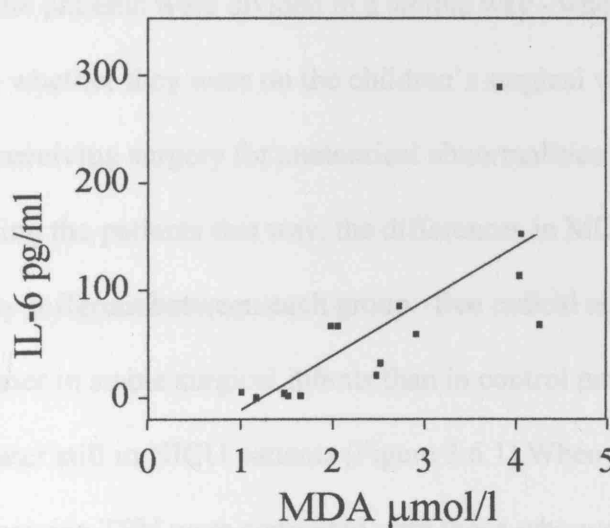


Figure 3.6.3 The relationship between MDA concentrations and a) TNF- α ($r=0.54$; $p<0.05$) and b) IL-6 ($r=0.74$; $p<0.001$) in patients not receiving TPN (Groups 1, 2 and 4).

5. Discussion

The purpose of this study was to explore the possibility that free radical activity may be related to the severity of illness in the patients in these studies. If sicker patients are receiving TPN and if free radical activity is also increased in this group because of their illness then the results of other studies, particularly Study 2 (where MDA concentrations in patients receiving TPN were compared with those who were not), may be brought into question. In addition, although increased free radical activity has been documented in many disorders and has been correlated with adverse outcomes in neonates (Inder 1994), there have not been any studies to determine whether there is a relationship between free radical activity and illness severity.

Initially the patients were divided in a simple way - whether they required intensive care or whether they were on the children's surgical ward. A group of control patients receiving surgery for anatomical abnormalities was also recruited. Dividing the patients this way, the differences in MDA concentrations were significantly different between each group: free radical activity was significantly greater in stable surgical infants than in control patients and was significantly greater still in NICU patients (Figure 3.6.1). When the patients in each location receiving TPN were compared with those who were not, the patients receiving TPN had significantly greater MDA concentrations (Figure 3.6.2). The patients in NICU receiving TPN were no sicker (as measured by cytokine levels and the PRISM score) than those who were not.

The control blood samples were obtained from 8 infants undergoing surgery for anatomical abnormalities who were systemically well. Differences between assay methods make comparison of MDA results between studies in the

literature difficult to interpret. Despite this the mean MDA result from the controls in the present study ($1.74 \mu\text{mol/l}$) was similar to that obtained by other investigators (e.g. $1.64 \mu\text{mol/l}$, Inder 1996).

Previous studies into free radical activity during TPN have not attempted to separate out the effect of degree of illness from the effect of administering intravenous nutrition (e.g. Wispe et al 1985; Pitkanen et al 1991). The administration of TPN to both stable surgical patients in the ward and patients in the intensive care unit resulted in a further significant increase in free radical activity, over and above the increase associated with their illness. There was no difference in the clinical characteristics between patients on the ward receiving TPN and those who were not. However the patients receiving TPN on NICU had a significantly lower gestational age and weight and a higher postnatal age at the time of the study than their counterparts receiving other forms of nutrition (Table 4.6.1). It is therefore possible that the observed difference in MDA may not be exclusively due to the effect of parenteral nutrition, but may be related to gestational age, weight or postnatal age. However this seems unlikely because MDA did not correlate with body weight, gestational age or postnatal age in any patient group, or in other studies (Yigit et al 1998).

A number of illness severity scores have been developed for patients receiving intensive care. This has been in order to assist in predicting outcome and comparing the effects of therapeutic interventions. The PRISM score is derived from 14 physiological and biochemical variables which are routinely monitored during intensive care. There was no correlation between the PRISM score and MDA concentrations in our study. However, when illness severity was

assessed more simply on the basis of whether the patient required ward care or intensive care, there was a significant difference between MDA concentrations in the two groups of patients (Figure 4.6.1³).

Cytokines are a structurally diverse group of proteins responsible for initiating and controlling the inflammatory response. TNF- α and IL-6 are two major pro-inflammatory cytokines which are released during infection by activated monocytes, macrophages and lymphocytes. They act at both the site of an infection, to activate leucocytes and systemically, to produce fever and promote acute phase protein synthesis. Cytokine levels have been correlated with clinical severity of illness in children with severe infections (Bhutta et al 1997; van Deuren et al 1995) and with the PRISM score in children with septic shock (Hazelzet et al 1994). TNF- α enhances superoxide production by neutrophils (Jersmann et al 1998) and TNF- α mediated cytotoxicity is associated with free radical generation (Larrick & Wright 1990). However contrasting findings suggest that TNF has free radical scavenging properties in cancer cells (Matsubara et al 1997). There are no studies relating plasma cytokines with severity of illness in newborn infants.

There was no correlation between MDA and cytokine concentrations or the PRISM score when the results of all the patients in the study were analysed as a single group. However when the patients not receiving TPN were analysed separately, there was a significant correlation between MDA and both TNF- α and IL-6. Previous studies demonstrated that TPN administration causes excess free radical production. Therefore any relationship between cytokines and MDA may be obscured in patients on TPN.

6. Conclusion

Free radical activity is increased in hospitalised, stable newborn infants compared with controls undergoing elective surgery. Free radical activity is even greater in those on NICU. The administration of TPN is associated with an additional elevation of free radical activity. Free radical production increases during critical illness and MDA concentrations correlate with TNF- α and IL-6 in patients not receiving TPN.

3.7 DOES ENTERAL FEEDING INFLUENCE FREE RADICAL ACTIVITY IN INFANTS RECEIVING TPN

1. Introduction

Some of the complications of TPN including biliary stasis (Jawaheer 1988), reduced immune function (Okada et al 1988) and reduced intestinal mucosal integrity are known to respond to small amounts of enteral feeding (Dunn et al 1988, Sax et al 1996).

The effect of partial enteral feeding on free radical activity in patients receiving TPN has not previously been examined.

2. Aim

The aim of this study was to investigate whether enteral feeding during TPN affected free radical activity.

3. Methods

This study was performed by comparing the plasma MDA concentrations in thirty one patients receiving TPN with that in nine patients who were receiving parenteral nutrition with some enteral feeding. The enteral feed was given as expressed breast milk or infant formula feed.

4. Results

There were no significant differences in the clinical characteristics of these two groups of patients (Table 3.7.1). The median enteral caloric intake was 11% (3-24%) of the total caloric intake in infants who received enteral

feeds. The MDA concentration was 5.23 μ mol/l (2.88-9.34 μ mol/l) in patients receiving total parenteral nutrition and 5.27 μ mol/l (3.19-8.89 μ mol/l) in patients receiving PN with partial enteral feeding (Figure 3.7.1). There was no significant difference in MDA concentrations between the two groups. There was no correlation between the percentage of calories taken enterally and MDA concentrations.

	TPN n=22	PN/EN n=9
Gestation (weeks)	34 (23-40)	36 (30-40)
Weight (kg)	2.37 (0.65-3.72)	3.00 (1.9-6.0)
Age (days)	28 (9-328)	63 (7-231)
Enteral calories (%)	0	11 (3-24)
MDA (μmol/l)	5.23 (2.88-9.34)	5.27 (3.19-8.89)

Table 3.7.1 Gestation, weight, age, percentage of enteral calories and plasma

MDA of patients in Study 7. Data are expressed as mean (range).

PN = parenteral nutrition

EN = enteral nutrition

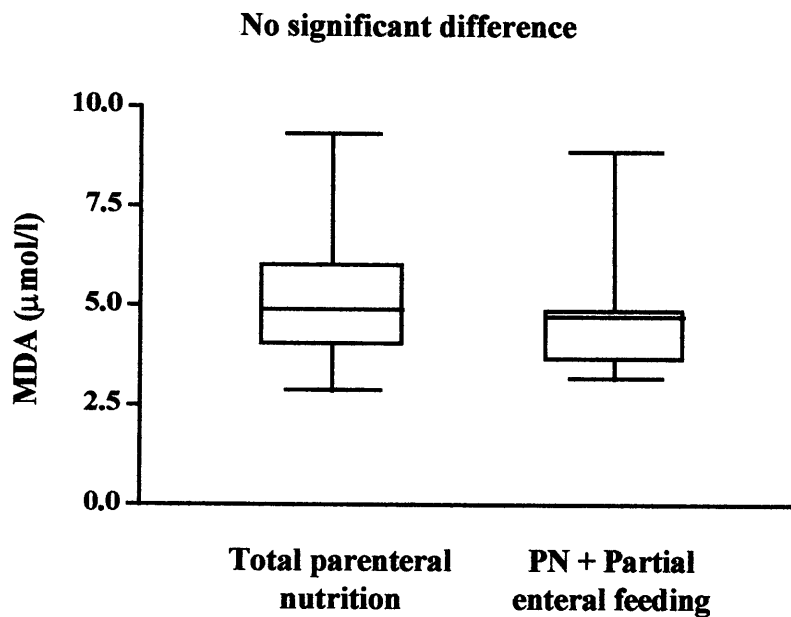


Figure 3.7.1 Comparison of MDA concentrations in patients receiving either TPN alone or with added enteral feeding.

The box extends from the 25th percentile to the 75th percentile, the horizontal line is at the median the whiskers show the range.

5. Discussion

It is conceivable that enteral feeding during TPN may influence free radical production by various mechanisms. If enteral feeding resulted in a decrease in the utilization of infused lipids then an increase in free radical activity may occur. Alternatively, by providing antioxidants such as vitamin E, enteral feeding may be associated with a decrease in free radical activity.

There was no difference in the plasma MDA concentrations between the two groups in this study. It is therefore possible that partial enteral feeding has no influence on TPN associated free radical activity. However in this study only the effect of minimal enteral feeding (mean 11% of total caloric intake) was

tested. It is possible that further increasing the percentage of calories given enterally may result in a change in free radical production.

CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

4. CONCLUSIONS AND SUGGESTIONS FOR FURTHER STUDIES

The studies I have undertaken were designed to answer some questions arising from the observation that free radical activity was increased during parenteral nutrition therapy.

Free radical mediated cell damage has been implicated in the pathogenesis of several neonatal disorders, including bronchopulmonary dysplasia, retinopathy of prematurity, necrotizing enterocolitis and intraventricular haemorrhage (Evans et al 1986; Clark et al 1988; Grace 1994). The administration of intravenous lipid emulsions is associated with an increase in indices of free radical activity (Wispe et al 1985; Pitkanen et al 1991). Although the administration of parenteral nutrition is not directly linked to all of these disorders, it is possible that this form of therapy may exacerbate free radical formation in these conditions. It is also possible that the pathogenesis of some of the complications associated with lipid emulsions, such as chronic lung disease, may be due to free radical mediated mechanisms.

The first study confirmed that the products of free radical activity were present in the Intralipid solution. The second study demonstrated that the indices of free radical activity were greater in patients receiving TPN and the third showed that MDA decreased in response to stopping the lipid infusion.

Following on from these studies I sought means of reducing lipid derived free radical concentrations while continuing to administer intravenous lipid. In

the fourth study the promotion of lipid oxidation by decreasing the carbohydrate administered was associated with a decrease in MDA.

In Study 5 the administration of a mixed medium and long chain triglyceride emulsion was associated with an increase in the free radical indices expired breath pentane and MDA. This unexpected effect may have been due to the stimulatory effect of MCTs on neutrophil free radical production, or the greater availability of under-used LCTs to participate in free radical formation.

Increased free radical activity has been associated with a poor clinical outcome, but not previously with measures of illness severity. In Study 6 I measured indices of free radical activity to see whether there was a correlation with the PRISM illness severity score, the white blood cell count and the plasma concentrations of the cytokines TNF- α and IL-6. There was no correlation between plasma MDA concentrations and the PRISM score, white cell count or cytokine concentrations in the whole group of patients. However when the results from patients not receiving TPN were considered, MDA concentrations showed a positive correlation with TNF- α .

The provision of small amounts of enteral feeding has been shown to ameliorate some of the complications of TPN. No difference in free radical activity was observed in a group of patients receiving enteral feeding in Study 7.

These clinical studies have addressed the problem of TPN derived free radicals by manipulating the lipid and carbohydrate in the TPN regimen. Another approach to the problem is the addition of antioxidants to the TPN solutions. This has been performed *in vitro* with conflicting results. The addition of multivitamin preparations to lipid emulsions was found to significantly increase lipid peroxidation in one study (Lavoie et al 1997) and to protect against peroxide formation in another (Silvers et al 2001). Clinical studies where antioxidant supplements are given to patients receiving TPN have not been performed. The effect of providing additional antioxidants (for example, vitamin E) could be assessed in two phase studies similar to those described in this thesis. If antioxidant supplementation were to cause a decrease TPN derived free radicals, their provision may be an important measure in preventing TPN related complications.

Structured lipids are triglycerides which may possess short, medium or long chain fatty acids on a single glycerol backbone. Originally created by random re-esterification of mixtures of triglycerides, structured lipids may now be manufactured to contain specific combinations of fatty acids (Stein 1999). Advantages suggested include increased fat oxidation, improved protein metabolism and a decreased effect on the reticuloendothelial system compared with LCT (Sandstrom et al 1995; Ekman et al 1987). Studies of free radical formation during infusion with structured lipids may permit the identification of triglycerides which are associated with the least free radical activity.

An increase in the indices of free radical activity has been correlated with an increased incidence of retinopathy of prematurity and chronic lung disease in premature neonates (Inder et al 1996; Nycyk et al 1998). Separate studies have suggested a link between these disorders and intravenous lipid administration (Chye et al 1999; Hammerman & Aramburo 1988). Further studies would be valuable to investigate whether the administration of TPN led to increased free radical formation and increased incidence and severity of these conditions in the same group of patients. A positive association would be strong evidence that TPN associated free radical activity is a clinical problem which should be addressed.

This thesis has described associations between TPN administration and free radical production, and between free radical activity and severity of illness. Studies to investigate whether free radical formation is actually causative of disease, and by what mechanism, may in the future offer the greatest hope of finding a solution to the problem of free radical mediated disorders in clinical practice.

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APPENDIX:
ADDITIONS AND AMMENDMENTS

Additional comments

p. 66

The retention is a measure of the speed at which a substance moves in a chromatographic system. The retention time is the time between injection and detection. Retention times are very sensitive to the conditions (flow rate of carrier gas, column temperature, sample size, etc.) used at the time the chromatogram was performed.

p. 76

The PRISM score is a pseudo-quantitative measure of illness derived from abnormalities of 14 physiological variables. These include those measured at the bedside such as heart rate and blood pressure, those measured in the laboratory such as plasma electrolytes, and more subjective “measurements” such as pupil size and Glasgow coma score. Derangements of these variables are given a number from 1 to 10, where a higher number is assigned to a more severe derangement. The PRISM score is the sum of these numbers, which may be from 0 to 76. The risk of mortality prediction is calculated by correcting the PRISM score for age and operative status.

p. 138

A possible relationship between MDA, disease severity and cytokines may be obscured in various ways in patients receiving TPN. The administration of TPN may affect some of the physiological and biochemical parameters used for the PRISM score. The effect of TPN could be tested in a study similar to Experiment 3, but where the TPN, not just the lipid component, is stopped for a period of time. Patients on TPN might have had other pathology resulting in a different cytokine response. This would be difficult to control for and could not be eliminated by stopping the TPN.

Results as mean \pm SD or median (range)

p. 86

Normally distributed data are presented as the median \pm the standard deviation (SD)

p. 89

MDA $8.69\mu\text{mol/l} \pm 3.48\mu\text{mol/l}$, LOOH $19.25\mu\text{mol/l} \pm 6.81\mu\text{mol/l}$

p. 97

The mean plasma MDA concentration was $5.27\mu\text{mol/l} \pm 1.87\mu\text{mol/l}$ in the group receiving TPN and $2.85\mu\text{mol/l} \pm 1.39\mu\text{mol/l}$ in the group not receiving TPN. This difference was significant at the $p < 0.0001$ level (Figure 3.2.1).

Lipid peroxides were measured in 20 of the patients not receiving TPN and 6 patients who were on TPN. The plasma lipid peroxide concentration was $4.39\mu\text{mol/l} \pm 3.29\mu\text{mol/l}$ in the group receiving TPN and $2.85\mu\text{mol/l} \pm 1.66\mu\text{mol/l}$ in the group not receiving TPN.

	Enteral feeding/i.v. crystalloid	TPN
	n=29	n=31
	mean \pm SD	mean \pm SD
Gestation (weeks)	38 \pm 3	34 \pm 4*
Age (days)	54 \pm 77	67 \pm 77
Weight (kg)	3.9 \pm 1.58	2.8 \pm 1.18**

Table 3.2.1 Gestation, age and weight of patients in Study 2

Significant difference, *p<0.0001, **p=0.001

Data are expressed as median (range)

Data are expressed as median (range).

	TPN n=22	PN/EN n=9
Gestation (weeks)	34 (23-40)	36 (30-40)
Weight (kg)	2.37 (0.65-3.72)	3.00 (1.9-6.0)
Age (days)	28 (9-328)	63 (7-231)
Enteral calories (%)	0	11 (3-24)
MDA (μmol/l)	4.92 (2.88-9.34)	4.78 (3.19-8.89)

Table 3.7.1 Gestation, weight, age, percentage of enteral calories and plasma MDA of patients in Study 7. Data are expressed as median (range).

PN = parenteral nutrition

EN = enteral nutrition

PATIENT INFORMATION DOCUMENT

Research Project

Metabolism of intravenous fat emulsion: oxidation, deposition, and generation of free radicals

We would like to ask your permission to include your child in this project.

Aim of the study

The aim of this project is to improve the composition of the special diet that infants and children receive when they are fed by drip. In particular we are interested to know how much of the fat given by drip is used to produce the energy required to support life (i.e. heart, liver, brain and the lungs function) and how much is stored in the body. We are also interested in finding out the levels of chemicals called free radicals when the drip feed is being administered.

Why is the study being done?

Infants and children with gut problems often cannot be fed orally and require a special diet that is given by drip. This special diet is called parenteral nutrition and is made of sugar, protein and fat. The amount of fat that infants and children need is not known. Moreover it is not known how the body of these small patients handle the fat given by drip.

How is the study to be done?

The treatment plan of your child will not be altered. Your child will be studied when he/she receiving parenteral nutrition, over a period of up to six days. In our study, three slightly different nutrition mixtures will be used. The mixtures will differ in the amount of sugar and fat they contain, and each will be given for two days at a time. In order to measure the proportion of fat oxidised to produce the energy needed for maintaining the body functions and the amount of fat deposited in the body for growth we need to measure the waste products of fat in the blood and the exhaled breath.

The air that your child is breathing in and out will be analysed continuously for 2 hours by an instrument called an indirect calorimeter to calculate the amount of energy consumed in the body. If your child is requiring assistance to breath the air will be collected from the breathing machine. If he/she is spontaneously breathing, the air will be collected from a transparent and ventilated hood positioned around the head. This system has been used by the applicants extensively in the past. It does not cause any discomfort and does not produce any complications.

In order to measure free radical production one small blood samples (1/3 of a teaspoon) and 3 samples of the expired breath will be collected for further analysis on each day of the study. Blood samples will be taken from a vein or a heel prick. Breath samples will taken using a small bag will be kept near the mouth of your child for approximately one minute. This new method of collecting breath samples -as been used by the investigators in other projects with no discomfort the patients.

What are the risks and discomforts?

There are no risks in participating in this study.

Measuring expired breath and collecting breath samples does not cause any discomfort.

Blood samples will be obtained at the same time as those routinely collected during your childs care, therefore no additional discomfort will result.

What are the potential benefits?

There are no potential benefits for your child in participating in this study. However this study will help us in design the ideal parenteral nutrition for infants and children who will need this type of feeding in the future.

Who will have access to the case/research records?

Only the researchers and a representative of the Research Ethics Committee will have access to the data collected during this trial.

This research has been approved by an independent Research Ethics Committee who believe that it is of minimal risk to your child. However, research can carry unforeseen risks and we want you to be informed of your rights in the unlikely event that any harm should occur as a result of taking part in this trial.

No special compensation arrangements have been made for this project but you have the right to claim damages in a court of law. This will require you to prove a fault on the part of the Hospital and/or any manufacturer involved.

Do I have to take part in this study?

If you decide now or at a later stage that you do not wish your child to participate in this research project, that is entirely your right and will not in any way prejudice any present or future treatment.

Who do I speak to if problems arise?

If you have any complaints about the way in which this research project has been, or is being, conducted, please in the first instance discuss them with the researcher. If the problems are not resolved, or if you wish to comment in any other way,

please contact the Chairman of the Research Ethics Committee, by post, via the R&D office, Institute of Child Health, 30 Guilford Street, London WC1N 1EH or, if urgent, contact the Committee Administration by telephone on extension 7777 who will try to sort matters out for you.

Details of how to contact the researchers:

Mr Rai Basu. Research fellow. Institute of Child Health Tel. No

Mr A Pierro, Senior Lecturer in Paediatric surgery, Institute of Child Health
Tel.

Great Ormond Street Hospital for Children NHS Trust and Institute of Child
Health Research Ethics Committee

Consent Form for PARENTS OR GUARDIANS of Children Participating in
Research Studies

95SG49 Metabolism of intravenous fat emulsion: oxidation, deposition and
generation of free radicals.

NOTES FOR PARENTS OR GUARDIANS:

1. Your child has been asked to take part in a research study. The person organising that study is responsible for explaining the project to you before you give consent.
2. Please ask the researcher any questions you may have about this project, before you decide whether you wish to participate.
3. If you decide, now or at any other stage, that you do not wish your child to participate in the research project, that is entirely your right, and if your child is a patient it will not in any way prejudice any present or future treatment.
4. You will be given an information sheet which describes the research project. This information sheet is for you to keep and refer to. Please read it carefully.
5. If you have any complaints about the way in which this research project has been, or is being conducted, please, in the first instance, discuss them with the researcher. If the problems are not resolved, or you wish to comment in any other way, please contact the Chairman of the Research Ethics Committee, by post via The Research and Development Office, Institute of Child Health, 30 Guilford Street, London, WC1N 1EH or if urgent, by telephone on 071 242 9789 ex 2620 and the committee administration will put you in contact with him.

CONSENT

I/We _____, being the parent(s)/guardian(s) of
_____ agree that the Research Project named above has been
explained to me to my/our satisfaction, and I/We give permission for our child to take
part in this study. I/We have read both the notes written above and the Information Sheet
provided, and understand what the research study involves.

SIGNED (Parent (s)/Guardian (s)) _____

SIGNED (Researcher) _____